TARGETING OF A MITOCHONDRIAL INTERMEMBRANE SPACE PROTEIN: FLAVOCYTOCHROME b_2

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To Mum, Dad, Graham and Gillian

DECLARATION.

I hereby declare that this thesis has been composed by myself and that all the work described herein is my own.

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ABSTRACT.

During their biogenesis, mitochondria import approximately 90% of their polypeptides from the cytoplasm. The majority of these proteins are synthesised as larger molecular weight precursors containing a targeting signal or presequence at their N-terminus. This presequence, usually 20-80 amino acids in length, directs the precursor to the mitochondrion and is proteolytically removed during the import process. While recent studies have determined many of the features associated with the import process, current work is focused on the molecular characterisation of components of the import apparatus and of precursor proteins themselves.

The work presented in this thesis describes an attempt to generate large quantities of the purified precursor of flavocytochrome b_2 a, mitochondrial intermembrane space protein from the yeast *Saccharomyces cerevisiae*, by its high-level expression in both yeast and *Escherichia coli*. A comparison between the expression of this precursor and the corresponding mature protein on the effect of growth of *E.coli* is made and the initial steps involved in purifying the precursor from the bacterium are described.

In addition, the first of two proteolytic cleavage sites is identified by constructing a modified form of the precursor protein by site-directed mutagenesis of the *CYB2* gene. This modified form accumulates in yeast mitochondria as an intermediate molecular weight species and the first cleavage site was identified by determining the N-terminal amino acid sequence of the intermediate form.

Finally, the targeting abilities of the remaining N-terminal presequence after the first cleavage has occurred are examined and the results discussed in relation to theories on the evolution of protein targeting pathways within mitochondria.

CONTENTS.

CHAPTER 1

GENERAL INTRODUCTION.

1.1. Protein traffic in eukaryotic cells.

Eukaryotic cells, unlike their prokaryotic ancestors, are characterised by a high degree of internal structure derived from the delineation of intracellular compartments or organelles by membranes. Each organelle is characterised by a specific subset of proteins and therefore by a specific group of biochemical functions.

Figure 1.1. illustrates some of the key organelles of a typical eukaryotic cell and table 1., the main functions of each. The biophysical and functional integrity of each organelle is maintained by its surface membrane which presents a permeability barrier and thus enables the segregation of those molecules required to carry out a given specific set of reactions. Both surface and intraorganellar membranes also provide a framework on which groups of enzymes and enzyme complexes can be grouped together such that co-ordinated biochemical reactions can occur with greater efficiency.

The permeability barrier created by organelle surface membranes also presents a problem when considering their biogenesis. Firstly, how do proteins which are synthesised on cytoplasmic ribosomes find the organelle in which they must assume their function and secondly, how then can a protein cross the hydrophobic core of a lipid bilayer and enter the lumenal space during transfer from one aqueous compartment to another. The targeting of a protein to a particular organelle is clearly specific, (the enzymes responsible for DNA replication are found in the nucleus and not in the endoplasmic reticulum), therefore all proteins except those remaining in the cytoplasm must contain, at least transiently, information relating to their specific subcellular location. This movement of proteins within eukaryotic cells is referred to as protein traffic and the information guiding a protein to its location as a targeting signal.

1.2. The secretory pathway.

The main destinations of non-cytoplasmic polypeptides in a eukaryotic cell are the secretory pathway, beginning with the targeting of a

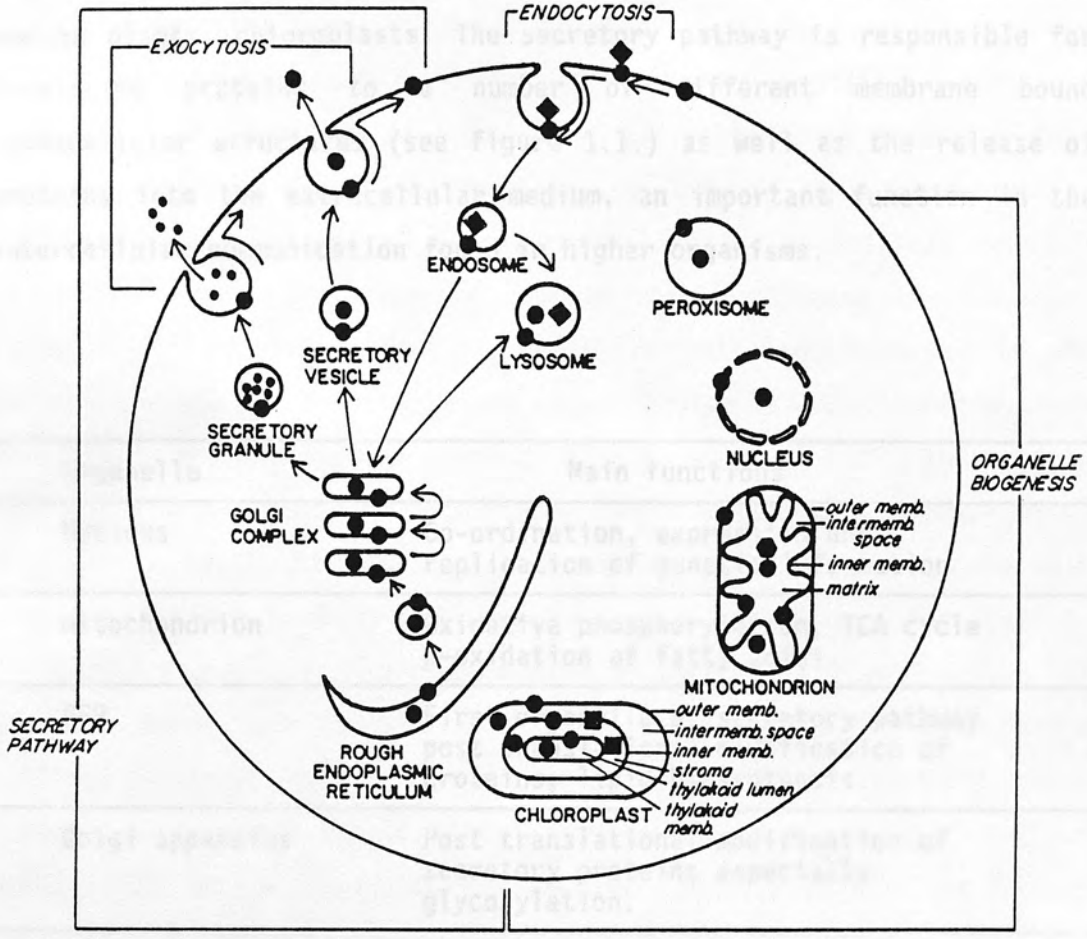

Figure 1.1.

The main organelles of the eukaryotic cell and principle features associated with protein traffic.

protein to the membrane of the rough endoplasmic reticulum (RER), or one of the non-secretory organelles, mitochondria, nuclei, peroxisomes and in plants, chloroplasts. The secretory pathway is responsible for localising proteins to a number of different membrane bound intracellular structures (see figure 1.1.) as well as the release of proteins into the extracellular medium, an important function in the intercellular communication found in higher organisms.

Organelle	Main functions
Nucleus	Co-ordination, expression and replication of genetic information
Mitochondrion	Oxidative phosphorylation, TCA cycle β–oxidation of fatty acids.
RER	First organelle of secretory pathway post translational modification of proteins, lipid biosynthesis.
Golgi apparatus	Post translational modification of secretory proteins especially glycosylation.
Secretory vesicle	Final stage of secretory pathway delivery of protein to extracellular medium.
Lysosome	Storage of hydrolytic enzymes for degradation of macromolecules.
Peroxisome	Storage of enzymes involved in oxygen metabolism e.g. catalase.

Table 1.
Principle functions of the major organelles of eukaryotic cells.

The targeting signals responsible for directing proteins to the RER membrane were the first involved in intracellular protein traffic to be

recognised. Milstein (1972) noticed that secreted immunoglobulin chains were shorter than the same polypeptides synthesised *in vitro*. These observations were extended and developed by Blobel and Dobberstein (1975) who observed that precursors of secretory proteins synthesised *in vitro* in the presence of RER-derived microsomes were processed to a shorter, mature form and imported into the lumen of the microsomes. The observed processing was due to the removal of a sequence of N-terminal amino acids from the precursor protein which according to the above authors was responsible for directing the attached precursor to and through the RER membrane whereupon it was proteolytically removed. Such N-terminal sequences were termed signal peptides and the signal hypothesis was proposed to account for its involvement in the targeting of proteins to the RER membrane. Subsequent to this early work a vast amount of information on the secretory pathway has been forthcoming. The signal peptides from a great number of proteins targeted to the RER membrane have been characterised and comparisons made, von Heijne (1986a). Although no conserved primary structure is apparent, some features are common to all signal peptides. They are generally 18-25 amino acids long and consist of a hydrophobic core. At the extreme N-terminus positively charged residues are common whereas at their junction with the mature protein acidic residues are often found. Signal peptides are however not obligatorily N-terminal since fusing another polypeptide to the N-terminus of certain secretory protein precursors does not impair signal peptide function (Simon *et al.*, 1987). Only one secretory protein discovered so far, ovalbumin, possesses an N-terminal signal peptide which is not proteolytically removed upon translocation across the RER membrane (Tabe et al., 1984)
The identification of a ribonucleoprotein complex termed the signal recognition particle (SRP) was a key development in extension of the signal hypothesis. SRP was observed to be required for the import of secretory protein precursors *in vitro* (Warren and Dobberstein, 1978). Subsequent investigation revealed that SRP is responsible for a reduction in the rate of translational elongation of secretory proteins

by binding to the signal peptide of the nascent protein. The SRP ribosome complex then interacts with a receptor protein on the surface of the RER, an interaction which results in the disassociation of the SRP and ribosome and the resumption of elongation of the secretory protein probably coupled to its transfer through a hydrophilic channel across the RER membrane. This co-translational mode of protein translocation is significant since it suggests a mechanism whereby the folding or more significantly the misfolding of proteins is prevented before they have to cross the lipid bilayer and assume their functional location. A large volume of experimental data exists to describe the complex biochemical interactions involved in the secretory pathway and significant evidence exists to indicate that the basic scheme of secretory protein targeting outlined above is not universally applicable but a more detailed analysis of the secretory pathway is outwith the scope of this introduction.

1.3. Targeting of proteins to non-secretory organelles.

Newly synthesised proteins not destined for the secretory pathway must further distinguish between several organelles during their correct intracellular localisation. Of these, targeting to the mitochondria and chloroplasts is best understood and is discussed more fully in the following sections.

Transport of proteins to the nucleus is less well understood. De Robertis *et al.* (1978) proposed that a specific sub-set of nuclear proteins was maintained by selective retention. This proposal was then backed up by the observation that the vast majority of nuclear proteins remained within the organelle after the disruption of the nuclear membrane with a glass needle (Feldherr and Ogburn, 1980). Studies by Dingwall *et al.* (1982) however suggested that a specific sequence of amino acids was required for the nuclear localisation of nucleoplasmin. Gene fusion techniques were then employed in an attempt to define the nuclear targeting signal. Hall *et al.* (1984) fused portions of the nuclear located MATα2 protein from yeast to β-galactosidase and found

that the nuclear targeting signal was located within the extreme N-terminal 13 residues. In a similar experiment, Kalderon *et al.* (1984) identified the nuclear targeting signal of the SV40 large T antigen as a sequence of residues near the middle of the polypeptide chain. Subsequent studies have confirmed that nuclear targeting signals may be located anywhere along the length of a polypeptide chain but are probably always exposed at the surface of the protein. As with the signal peptides of secretory proteins, nuclear targeting signals do not share a common amino acid sequence, however most tend to be between 5-10 residues in length and contain several lysine and arginine residues. It appears that proteins enter the nucleus at specific sites corresponding to the nuclear pore. Some evidence exists to suggest that proteins may bind to receptors and queue up to cross the nuclear pore (Feldherr *et al.*, 1984).

The import of proteins into peroxisomes is perhaps least well understood. These membrane bound organelles were originally thought to form by budding off from the RER membrane but the majority of evidence now favours the post translational import of proteins during their biogenesis. The nature of the peroxisomal targeting signal is not well understood. As with secretory and nuclear proteins gene fusion experiments have led to the identification of a putative targeting sequence. Gould *et al.* (1987) found that the extreme C-terminal 13 residues of the firefly luciferase protein could direct DHFR to the peroxisome. This sequence was unusually rich in lysine and neutral or hydrophobic residues but some peroxisomal proteins do not contain this sequence. A specific receptor is also thought to exist on the peroxisomal surface since peroxisomal proteins synthesised *in vitro* are targeted efficiently even in the presence of mitochondria (Imanaka *et al.*, 1987).

1.4. Import of proteins into mitochondria.

Mitochondria, like chloroplasts, possess the capacity to synthesise their own proteins, however the vast majority of mitochondrial proteins

are nuclear encoded and synthesised on cytoplasmic ribosomes. These proteins then have to find the correct organelle, cross one or both membranes and assemble into functional enzymes often forming oligomeric complexes with mitochondrially encoded polypeptides. The last 10 years has seen a wealth of information generated on various features of this targeting.

1.4.1. Co- versus post translational import.

Experimental evidence to suggest that proteins are imported into mitochondria by both co- and post translational mechanisms has been presented. Since the targeting signals on mitochondrial precursors are located at the N-terminus (see next section) it is feasible to assume that on emergence from the ribosome the nascent chain-ribosome complex could be targeted to the mitochondrial surface whereupon the elongation of the nascent chain across the mitochondrial membrane would occur. This idea was supported by the observation that 80S ribosomes were co-isolated with yeast mitochondria (Kellems *et al.*, 1974). The majority of these ribosomes could only be released after treatment with puromycin suggesting that the ribosomes were anchored to the mitochondria via the nascent chain. These mitochondrially associated ribosomes were enriched with mRNAs encoding mitochondrial proteins (Ades and Butow, 1980). Not all mitochondrial proteins however were preferentially synthesised on mitochondrially associated ribosomes. Suissa and Schatz (1982) observed that although approximately 60% of the translatable mRNA for the F_1ATPase β-subunit was bound to mitochondrially associated polysomes, more than 95% of cytochrome oxidase subunits V and VI and porin mRNA was bound to free polysomes. These experiments were performed in the presence of cycloheximide to permit the arrest of translation and enable nascent chains to bind irreversibly at the mitochondrial surface. The observed association of ribosomes with the mitochondrial surface may not therefore reflect the physiological situation.

A considerable amount of evidence exists to suggest that mitochondrial protein import occurs post translationally both *in vitro* and *in vivo*. Cytoplasmic pools of mitochondrial precursor proteins have been identified after pulse-labelling *Neurosporra crassa* (Hallermayer *et al.*, 1977) and yeast (Reid and Schatz, 1982b). Several studies have shown that import of precursors can occur *in vitro* in the absence of protein synthesis e.g. by the use of cycloheximide (Harmey *et al.*, 1977).

Taken together, the available data suggests that while some precursors might be imported in a co-translational manner *in vivo*, the mechanistic coupling of protein synthesis with translocation as with the majority of secretory proteins, is not obligatory. Recently Verner and Schatz (1987) reported that nascent mitochondrial precursors with attached tRNA were imported more efficiently than the corresponding completed polypeptides. The significance of this finding will be discussed in a later section.

1.4.2. Mitochondrial targeting signals.

Maccecchini *et al.* (1979) observed that mitochondrial proteins were smaller than their *in vitro* synthesised precursors. The N-terminal sequence analysis of both precursor and mature forms of mitochondrial polypeptides led to the identification of N-terminal presequences (Viebrock *et al.*, 1982) which were proteolytically removed during translocation across the mitochondrial membrane. The existence of N-terminal presequences was further evident when comparing mitochondrial and cytoplasmic isoenzymes. The yeast mitochondrial alcohol dehydrogenase isoenzyme (ADH3) contains an amino acid presequence not found on the corresponding cytoplasmic forms. When this sequence is removed by mutagenesis of the *ADH3* gene the protein is no longer targeted to the mitochondrion and remains in the cytoplasm (van Loon and Young, 1986).

1.4.3. Gene fusion experiments in mitochondrial targeting.

Gene fusion experiments to identify targeting signals have already been discussed in the context of nuclear and peroxisomal targeting. A large number of similar experiments have been performed to identify the targeting signals of mitochondrial precursors. In general, the cleavable N-terminal presequence from a mitochondrial precursor when fused to a non-mitochondrial protein e.g. DHFR, can direct the resultant fusion protein into the mitochondrion both *in vitro* and *in vivo*. The 32 amino acid presequence of both rat and human ornithine carbamyltransferase (OCT) directs a non-mitochondrial passenger protein into the mitochondria (Horwich *et al.*, 1985). Similarly the 25 amino acid N-terminal presequence of yeast cytochrome c oxidase subunit IV (COXIV) could direct DHFR to the mitochondria both *in vivo* and *in vitro* (Hurt *et al.*, 1984)

Gene fusion techniques have also been used to define the areas within a mitochondrial presequence which are responsible for targeting. Hurt *et al.* (1985) found that the first 12 amino acids from the COXIV presequence were sufficient to target DHFR to the mitochondrion both *in vitro* and *in vivo* although removal of the first 7 amino acids from the same presequence abolished targeting. Not all targeting signals reside at the extreme N-terminus however since Horwich *et al.* (1986) located the critical targeting information to residues 8-22 of the 32 amino acid presequence of rat OCT. The finding that not all of a mitochondrial presequence is required for targeting of a passenger protein raises the possibility that such sequences may be at least partially, functionally redundant. Bedwell *et al.* (1987) have proposed that other presequence segments can act cooperatively to acheive maximum efficiency of targeting *in vivo*.

Recently, a number of mitochondrial proteins have been shown to be able to be targeted to the mitochondria even in the absence of their N-terminal presequence (Volland and Urban-Grimal, 1987; Thompson and McAlister-Henn, 1989). The significance of these observations is unclear however it would appear that these proteins possess sequences

within their mature regions which can compensate for the lack of a presequence and interact with the necessary import machinery.

1.4.4. Properties of mitochondrial presequences.

The observation that mitochondrial presequences contain the information necessary to direct almost any protein to the mitochondrion prompted a search for the key elements involved in this targeting.

von Heijne (1986b) analysed 23 different mitochondrial presequences mainly from lower eukaryotes and reported the lack of any significant amino acid sequence homology. Presequences do however share a number of common characteristics, they contain a high proportion of positively charged amino acids, a correspondingly low proportion of negatively charged residues and a high content of hydroxylated amino acids. In particular, mitochondrial presequences are enriched in arginine, leucine and serine residues. A further common characteristic observed by the above authors was the ability of mitochondrial presequences to form α-helices with a high hydrophobic moment. Such amphiphilic helices should theoretically have a high affinity for negatively charged lipid bilayers. Roise *et al.* (1986) tested this last prediction by examining synthetic peptides corresponding to the COXIV presequence. Such peptides were observed to generate circular dichroism spectra characteristic of random coils when in aqueous solution but in the presence of detergent micelles adopted an α-helical conformation. The above workers further observed that presequence peptides could not only actively associate with lipid bilayers but could disrupt them. This disruption effect was enhanced if a transmembrane potential similar to that maintained across the mitochondrial inner membrane was applied. Significantly a C-terminally truncated form of the COXIV presequence could not disrupt lipid bilayers even in the presence of a transmembrane potential. Based on these observations the above workers proposed a model to describe the role of amphiphilic helices in translocation of a precursor protein across the mitochondrial inner membrane (figure 1.2.). Recently, Endo *et al.* (1989) have shown that

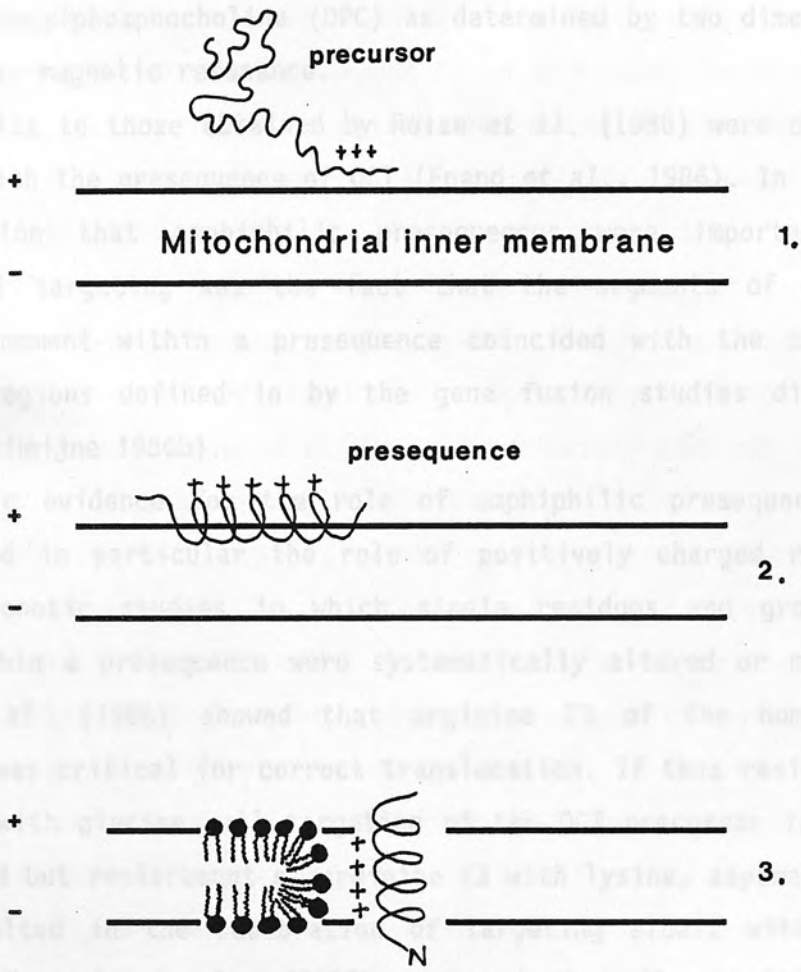

Figure 1.2.
Proposed interaction between a mitochondrial presequence and an energised mitochondrial inner membrane.
(Redrawn from Roise *et al.*, 1986)

(1) The loosely folded precursor binds to the mitochondrial surface. (2) The presequence interacts with membrane phospholipids resulting in formation of an amphiphilic α-helix, the hydrophobic face of which possibly buries into the lipid bilayer. (3) The presequence inserts across the lipid bilayer either through electrophoresis of the positive charges or orientation of the helical dipole or a combination of both.

the amino terminal half of a synthetic peptide corresponding to the COXIV presequence forms an α-helical structure in the presence of the detergent dodecylphosphocholine (DPC) as determined by two dimensional proton nuclear magnetic resonance.

Similar results to those obtained by Roise *et al.* (1986) were obtained in studies with the presequence of OCT (Epand *et al.*, 1986). In support of the notion that amphiphilic presequences were important for mitochondrial targeting was the fact that the segments of maximal hydrophobic moment within a presequence coincided with the critical functional regions defined in by the gene fusion studies discussed earlier (von Heijne 1986b).

More specific evidence for the role of amphiphilic presequences in targeting and in particular the role of positively charged residues came from genetic studies in which single residues and groups of residues within a presequence were systematically altered or removed. Horwich *et al.* (1986) showed that arginine 23 of the human OCT presequence was critical for correct translocation. If this residue was substituted with glycine, all targeting of the OCT precursor *in vitro* was abolished but replacement of arginine 23 with lysine, asparagine or alanine resulted in the restoration of targeting albeit with lower efficiency. Horwich *et al.*, (1986) proposed that the abolition of targeting upon the introduction of glycine at position 23 in the OCT presequence results not from the loss of a positive charge but rather caused by the disruption of local secondary structure due to the presence of the glycine.

The absolute requirements for mitochondrial targeting were further defined by Allison and Schatz (1986). These workers fused artificial presequences encoded by synthetic oligonucleotides to the N-terminus of the COXIV mature region and selected fusions which were imported into mitochondria *in vitro* and *in vivo*. The only common requirement for import was found to be an amphiphilic α-helical secondary structure even when presequences were composed entirely of serine, threonine and arginine. Roise *et al.* (1988) subsequently extended this definition to

include an amphiphilic β-sheet indicating that amphiphilicity and not a helical structure was the key factor .

The apparent abundance of amphiphilic α-helices even in random sequences (Flinta *et al.*, 1983) seemed to suggest that the structural requirements for mitochondrial targeting were low. This possibility was borne out by the observation that a significant proportion of random sequences generated from an *E.coli* DNA bank could encode a functional mitochondrial targeting sequence when fused to the N-terminus of the mature COXIV protein (Baker and Schatz, 1987). One further requirement of a mitochondrial targeting signal was recognized by Hurt and Schatz, (1987). These workers placed a DNA sequence encoding part of the DHFR polypeptide normally sequestered within the protein at the 5′ end of the DHFR coding sequence, the resulting polypeptide was found to be imported into mitochondria. The authors then proposed that several such cryptic mitochondrial signals might exist but could only be functional when placed at the surface of the protein (as is usually the case when localised at the extreme N-terminus).

Recently, von Heijne *et al.* (1989) re-examined a total of 37 mitochondrial presequences and proposed a specific structure whereby amino terminal and carboxyl terminal domains exist within a presequence, each with a characteristic hydrophobic moment. These authors also recognised a characteristic high content of proline and serine residues in the polypeptide chain immediately downstream of the site of proteolytic processing and proposed that such a feature, indicative of a loosely folded region of polypeptide, might be important in recognition of a cleavage site by the matrix protease.

1.4.5. Intramitochondrial sorting of imported proteins.

The features associated with mitochondrial targeting disscussed so far apply directly to those precursors destined for the mitochondrial matrix or matrix face of the inner mitochondrial membrane. Mitochondrial proteins may occupy other compartments within the

organelle (see fig 1.1). Do mitochondrial presequences specify intramitochondrial location of a protein?

The presequences of all imported proteins contain at the extreme N-terminus a targeting signal in keeping with the common characteristics already discussed so far (with a very few exceptions). Such signals are responsible for targeting proteins to the mitochondrial matrix or the matrix face of the mitochondrial inner membrane. Proteins destined for the intermembrane space contain in addition an extra signal within the presequence characterised by a sequence of uncharged amino acids which is responsible for their localisation in the intermembrane space (the targeting of proteins to the intermembrane space is discussed in detail in chapter 4).

Proteins destined for the outer membrane do not contain a cleavable presequence but their N-terminal portions closely resemble those of intermembrane space proteins. Hase *et al.* (1984) fused the first 41 amino acids from the yeast 70 kDa outer membrane protein to the N-terminus of *E.coli* β-galactosidase and found that the resulting fusion protein is located in the mitochondrial outer membrane with the same orientation as the authentic 70 kDa protein. Outer membrane proteins are thus believed to be anchored in the membrane and prevented from being completely translocated into the matrix by virtue of a hydrophobic stop-transfer domain adjacent to the matrix targeting signal (Hurt and van Loon, 1986).

Mitochondrial presequences or at least the N-terminus of mitochondrial proteins therefore contain the information necessary for correct intracellular and intraorganellar targeting.

1.4.6. Proteolytic processing of imported precursors.

Most imported mitochondrial precursors undergo the proteolytic removal of the N-terminal presequence during or immediately after translocation across the mitochondrial membrane. This processing is carried out on the matrix face of the mitochondrial inner membrane by a single soluble protease which requires divalent cations for its function (Böhni *et*

al., 1983). A single enzyme apparently possesses the ability to recognise a large number of different precursors. The fact that the matrix protease is present only in very small amounts made its characterisation difficult. Recently however two groups have independently purified the matrix processing activity from both *N.crassa* and *S.cerevisiae* (Hawlitschek *et al.*, 1988; Yang *et al.*, 1988). Both enzyme activities were observed to require the presence of two loosely associated, non-identical subunits. In the case of the protease from *N.crassa*, a 57 kDa protein was observed to process mitochondrial precursors *in vitro* at a low level. This protein was designated the matrix processing protease (MPP) (Hawlitschek *et al.*, 1988). Upon addition of a 52 kDa component however the rate of precursor processing by the MPP increased several fold. This non-catalytic subunit was designated the processing enhancing protein (PEP) and was required for efficient protease activity. The MPP was found to be a soluble matrix protein while approximately 75% PEP was associated with the mitochondrial inner membrane. Interestingly the two proteins were not present in stoichiometric amounts in mitochondrial extracts (PEP was present in some 15 fold molar excess over MPP in *N.crassa*) despite the fact that a single subunit of each was required for maximal processing activity.

The observation that two independent polypeptides were required for normal processing was interesting since Yaffe and Schatz (1984) had previously identified two temperature sensitive lethal mutations in yeast, *mas*1 and *mas*2, which interfere with mitochondrial precursor processing. Did these mutations correspond to the two subunits required for processing? Witte *et al.* (1988) subsequently cloned and sequenced the *mas*1 gene from yeast. Antibodies raised against MAS1 β-galactosidase fusion protein cross reacted with the PEP from *N.crassa* indicating that these proteins were probably functionally equivalent. These two proteins were also observed to exhibit 60% amino acid sequence identity. Using a different screening method, Pollock *et al.* (1988) have independently isolated two temperature sensitive lethal

mutations in yeast defective in precursor processing designated *mif*1 and *mif*2. The *mif*2 mutation was found, by genetic crossing to be in the same complementation group as *mas*1. This gene was observed to directly affect the proteolytic processing of precursors and hence to correspond to the MPP subunit. Sequence analysis of the *mif*2 (*mas*2) gene revealed a significant level of similarity between the protein sequences of MPP and PEP. This observation was surprising considering the difference in function and stoichiometry between the two proteins. Hawlitschek *et al.* (1988) have suggested that PEP might play a key role in protein import by recognising precursor proteins as they cross the mitochondrial inner membrane and presenting them to the MPP for processing. A previous observation that *mas*1 mutants failed to import several mitochondrial precursors (Yaffe, *et al.*,1985) is supportive of the notion that PEP might be essential for protein translocation as well as processing.

No clear recognition sequences for proteolytic processing have been identified. Nicholson and Neupert (1988) compared the amino acid sequences flanking the cleavage sites of a number of precursors but reported only weak sequence similarities. Other evidence exists to suggest that regions within the presequence are important in defining the site of processing. The precursor of coxIV is normally processed between residues 25 and 26 of the precursor. When only the first 22 amino acids are fused to DHFR the fusion protein is still imported and processed but between residues 17 and 18, despite the fact that the sequence of amino acids flanking the new cleavage site bears no resemblance to the authentic site (Hurt *et al.*, 1985). The processing of a COXIV-DHFR fusion protein by a solubilised matrix extract was blocked by small deletions at the extreme N-terminus (Hurt *et al.*, 1987). Amino acid sequences within the mature protein can also influence the site of processing. Nguyen *et al.* (1987) fused the first 60 amino acids from the rat OCT precursor to the N-terminus of asparagine synthase and observed correct processing resulting in removal of a 32 amino acid presequence. When only the first 37 amino acids were fused to asparagine synthase, however, processing was

observed to occur at a site 14 residues closer to the amino terminus. The combined data from these and similar studies strongly suggests that the site of processing of a mitochondrial precursor protein is dictated by the overall tertiary structure and not a localised sequence motif.

Proteolytic proccesing is however not obligatory for mitochondrial import. In addition to outer membrane proteins e.g. the 70 kDa protein, the ADP/ATP carrier protein of the mitochondrial inner membrane does not possess a cleavable presequence. It appears that this protein contains signals for its mitochondrial targeting within its polypeptide sequence some distance from the N-terminus (Smagula and Douglas, 1988). Cleavage at such an internal site might therefore render the protein non-functional. Cytochrome c also lacks a cleavable presequence but the import of this protein is thought not to be representative of the general case (see next section). Several workers have reported deletions or specific mutations within presequences which abolish processing but do not inhibit translocation across the mitochondrial membrane.

The reason for the proteolytic removal of mitochondrial presequences might be due to the potentially toxic nature of a high concentration of amphiphilic sequences within the mitochondrion (Roise and Schatz, 1988). The proteases involved in breakdown of such sequences after their removal from the precursor are however largely unidentified.

1.4.6. Mitochondrial import receptors.

The preceding sections have described how the information contained within a mitochondrial precursor protein is responsible for its targeting and import into mitochondria. In order for this information to be decoded biochemically, complementary components must exist on the mitochondrial surface. The existence of specific receptors on the mitochondrial surface had been proposed before many other import features had been recognised but until very recently no such molecules had been characterised. A great deal of experimental data has however been generated in support of the role of receptors. Gasser *et al.*

(1982) observed that isolated mitochondria treated with low concentrations of protease do not import or bind certain mitochondrial precursors. Binding of precursor proteins to the mitochondrial surface is specific (i.e. cytoplasmic proteins do not bind) and is dependent on a mitochondrial presequence as mature proteins or the intermediate form of an intermembrane space proteins do not bind to mitochondria (Riezman *et al.*, 1983a). The binding of mitochondrial precursors could occur in the absence of a transmembrane potential indicating that binding was independent of import (Pfanner and Neupert, 1985). Binding sites on the surface of mitochondria could be reconstituted into outer membrane vesicles (Riezman, 1983b) These had the same affinity for precursor proteins as whole mitochondria and were also sensitive to added protease. Gillespie *et al.* (1985) showed that the binding of a precursor to mitochondria could be inhibited by other precursor proteins and even by chemically synthesised presequence peptides, thereby indicating that more than one precursor could bind the same receptor and binding was competitive.

Given the very large number of imported polypeptides the idea that one receptor could recognise more than one precursor was not surprising but there is clearly more than one receptor. Currently at least four different classes of receptor are recognised (Pfanner *et al.*, 1988a), one each mediating import of cytochrome c, the ADP/ATP carrier of the mitochondrial inner membrane and the outer membrane porin with a further class responsible for import of precursors with cleavable N-terminal presequences.

The binding of proteins to mitochondria is best characterised for apocytochrome c although it appears that this protein undergoes a somewhat different import pathway to that of other mitochondrial proteins. Hennig *et al.*, (1983) showed that radiolabelled apocytochrome c could be displaced from mitochondria after binding (in the presence of deuterohemin to prevent import) by the addition of chemically synthesised apocytochrome c, thus demonstrating reversible, competitive binding. Apocytochrome c binding sites could be reconstituted into

liposomes and the receptor purified to homogeneity (Kohler *et al.*, 1987). This protein was found not to be a constituent of the outer membrane but was instead a soluble intermembrane space protein, despite the fact that it could compete with intact mitochondria for apocytochrome c binding. Nicholson *et al.* (1987) have suggested that the apocytochrome c binding protein acts by recognising the protein as it crosses the outer membrane, possibly by spontaneous insertion across the bilayer and promotes the binding of heme by a separate protein.

The binding of the ADP/ATP carrier of the inner mitochondrial membrane has been resolved into two distinct steps (Pfanner *et al.*, 1988a). Firstly, the precursor interacts with a protease sensitive receptor site on the mitochondrial surface where it remains itself accessible to externally added protease. The precursor then inserts into a protease-protected site after association with a second, protease protected component in the outer membrane. The import of the outer membrane porin also exhibits a similar two step mechanism during its import. Porin and the ADP/ATP carrier protein interact with a different protease-sensitive receptor site on the mitochondrial surface but the subsequent interaction of porin with the second, protease-protected outer membrane component could inhibit the second step of the ADP/ATP carrier protein import. These workers suggested that although both the ADP/ATP carrier protein and porin recognised separate receptors on the mitochondrial surface, their import pathways converged at a second protein. The observation that porin could compete with precursors destined for each submitochondrial location at this common component prompted these workers to define this component as the general insertion protein (GIP).

Despite this wealth of evidence to implicate receptors in the import process the unambigous identification of any candidates for such a role has proved difficult. A number of groups have shown that antibodies raised against different outer membrane components could inhibit import of precursors *in vitro*. In such an experiment, Ohba and Schatz, (1987)

identified a 45 kDa protein from the yeast mitochondrial outer membrane. Antibodies or Fab fragments against this protein caused a decrease in the level of import if mitochondria were first treated with protease. Similarly antibodies against a 19 kDa protein from *N.crassa* mitochondria designated MOM19 have been shown to inhibit precursor uptake *in vitro* even in the absence of any protease pretreatment (N.Pfanner, unpublished). Gillespie (1987) also reported that antibodies against a 30 kDa protein from mammalian mitochondria interact with a chemically synthesised mitochondrial presequence.

Recently Vestweber *et al.* (1989) employed a different strategy to identify a receptor from the outer membrane of yeast mitochondria. These workers constructed a chimaeric precursor protein consisting of a COXIV-DHFR fusion with the bovine pancreatic trypsin inhibitor (BPTI) chemically linked to the C-terminus of the DHFR moiety. This molecule had previously been observed to stick in the mitochondrial membrane at import sites due to the inability of the BPTI molecule to adopt a relaxed structure and hence be translocated across the mitochondrial membrane. By placing a photoreactive group in the link between the DHFR and BPTI domains a covalent bond could be formed between the chimaeric precursor and a protein at the import site upon illumination of mitochondria. In this way Vestweber *et al.* (1989) identified a 42 kDa protein as a component of the mitochondrial import site. Antibodies subsequently raised to this protein could inhibit the import of other mitochondrial precursor proteins even when antiserum was depleted of IgG specific for the 45 and 30 kDa proteins previously proposed to recognise imported proteins

The identification of this protein termed ISP42, represents the first positive identification of a protein at the mitochondrial surface essential for protein import. It is however possible and quite probable that several different proteins are involved in the recognition and subsequent translocation of precursors.

1.4.7. Energy requirements.

Binding of precursors to mitochondria occurs independently of subsequent import. One key difference between these two events is in the requirement of an energy source for the latter. Initially, ATP was believed to supply this energy but work by several groups has indicated a requirement for an electrochemical potential gradient (Schleyer *et al.*, 1982; Gasser *et al.*, 1982). Pfanner and Neupert (1985) showed that specifically a transmembrane electrical potential difference ($\Delta\psi$) and not the pH gradient was utilised. This observation was hardly surprising since ($\Delta\psi$) contributes the vast majority of the mitochondrial electrochemical potential under physiological conditions. Schleyer and Neupert (1985), by carefully resolving intermediate stages during precursor import, demonstrated that only the initial translocation of the presequence across the mitochondrial inner membrane was dependent on an energy supply; subsequent translocation of the mature polypeptide chain occurred independent of a ($\Delta\psi$). These experimental observations supported the theoretical proposal by von Heijne (1986b) that an electrical potential across the mitochondrial inner membrane (negative inside) could make the transfer of positive charges in a presequence across this membrane energetically favourable. In addition to the requirement for a transmembrane potential more recent experiments from several laboratories have shown that nucleoside triphosphates are also required for import of precursors (see e.g.Chen and Douglas, 1987). Specifically ATP or GTP were required and could not be replaced by their non-hydrolysable analogues, indicating that the hydrolysis of a high energy phosphate bond was essential. Pfanner *et al.* (1987a) showed that several steps during precursor import required NTPs but that the common feature of these steps appeared to be the translocation of precursor domains from the cytosol onto and across the mitochondrial surface. Lower levels of NTPs were required for formation of a translocation intermediate in which the presequence had been proteolytically removed but the bulk of the protein was exposed to the

cytoplasm whereas higher levels were required for complete translocation of the polypeptide across the bilayer.

Eilers and Schatz (1986) had previously observed that an artificial precursor protein could not be imported into mitochondria if it was first incubated with a ligand which binds tightly to the passenger protein. The authors proposed that ligand binding prevented the unwinding of the protein and that such a modification of protein conformation was essential for mitochondrial import. This proposal was not surprising since the uptake of fully folded proteins by mitochondria would require large pore structures on the mitochondrial surface. How such protein unfolding at a membrane surface was mediated was unclear, however Rothman and Kornberg (1986) suggested that ATP dependent "unfoldases" known to be involved in biochemical reactions which involved the relaxation of tertiary structure, could conceivably perform a similar role in mediating the unwinding of a precursor protein prior to translocation across a membrane. Did the requirement for ATP in mitochondrial protein import correlate with the unfolding of precursors prior to membrane translocation?

Pfanner *et al.*(1987a) demonstrated that an unfolded conformation of precursor proteins as determined by an increased sensitivity to proteinase K did correlate with the amount of ATP present in an *in vitro* import system. Furthermore when different passenger proteins were fused to a mitochondrial targeting signal different amounts of ATP were required to promote the unfolding of precursors at the mitochondrial surface reflecting the differences in size and complexity of folding between different passenger proteins. Further evidence of the role of ATP hydrolysis in unfolding precursor proteins came from the observation that nascent chains of mitochondrial precursors attached to tRNAs could be imported into mitochondria in the absence of NTPs (Verner and Schatz, 1987). These nascent chains were highly sensitive to proteinase K and could not bind ligand molecules unlike the full length precursor polypeptide, indicating that they already had a loosely folded conformation prior to incubation with mitochondria.

Further experiments showed that the same precursor protein partially denatured by 8M urea or destabilised by selective mutation of its gene could be imported into isolated mitochondria much more rapidly and at lower temperatures than the native protein (Eilers *et al.*, 1988; Vestweber and Schatz, 1988). The unfolding of precursor proteins was therefore not only dependent on NTPs but was rate limiting and cold sensitive during import into mitochondria.

The nature of the unfolding reaction is unclear. In particular it is not known if unfolding of precursors occurs in the cytoplasm or at the mitochondrial surface during translocation. Eilers *et al.* (1988) showed that the COXIV-DHFR precursor bound to the mitochondrial surface and was at least partly unfolded in the absence of NTPs. Subsequent translocation however was dependent on ATP hydrolysis. Deshaies *et al.* (1988) reported that a yeast mutant lacking certain members of a class of 70 kDa heat shock proteins (hsp70s) accumulated the unprocessed β-subunit of the mitochondrial F_1ATPase and the unprocessed prepro-α-factor, a secreted protein. Prepro-α-factor could be detected in the cytoplasm of mutant yeast cells but owing to the lability of the $F_1\beta$-precursor in cell lysates its location could not be determined. Since Pelham (1986) had already suggested that members of the hsp70 class were responsible for coupling ATP hydrolysis to the disruption of oligomeric or denatured aggregates, Deshaies *et al.* (1988) on the basis of the above observations, extended the proposed functions of hsp70s to include the modulating of secretory and mitochondrial precursor protein folding to maintain an import competent conformation. This claim was reinforced by the observation that purified hsp70 protein was required for precursor translocation *in vitro* (Chirico *et al.*, 1988) and the earlier finding that purified $F_1\beta$ could only be imported to isolated mitochondria in the presence of a soluble cytosolic factor (Ohta and Schatz, 1984).

Such a role for hsp70 proteins is consistent with that proposed for a number of proteins from different organisms classified as molecular chaperones or chaperonins (Ellis, 1987). These proteins have all been

shown to at least potentially play a role in protein translocation but may be involved in several intracellular processes. Their common denominator seems to be the ability to prevent the mis-folding or aggregation of a specific subset of proteins and thereby enable them to carry out their function (Hemmingsen *et al.*, 1988)

Whether proteins of the hsp70 class are responsible for the unfolding of mitochondrial precursors *in vivo* is not known. Their role might simply be to chaperone a precursor to the mitochondria where another, possibly membrane bound, protein might actively couple NTP hydrolysis with membrane translocation.

1.4.8. Overview.

Figure 1.3. illustrates some of the key features associated with uptake of a precursor protein by mitochondria. A feature generally associated with uptake of all mitochondrial precursors is their import at translocation contact sites where inner and outer membranes are closely opposed. Such sites were first visualised by transmission electron microscopy (Hackenbrock, 1968). Schleyer and Neupert (1985) later showed that a precursor could be trapped during import such that its presequence had been removed by the matrix protease whereas the bulk of the protein remained accessible to the cytoplasm. These observations suggested that precursors crossed both membranes simultaneously and not via a soluble intermembrane space intermediate. Recently the existence of translocation contact sites has been shown using protein A-gold labelling to tag the precursor of the β-subunit of F_1ATPase translocation of which had been blocked after binding to the mitochondrial surface by prior incubation with antibodies directed against the mature F_1ATPase β-subunit. When visualised by electron microscopy, the gold particles were exclusively localised in regions of contact between inner and outer membranes (Schwaiger *et* al., 1987).

The final step in the import of a mitochondrial protein is the formation of an active enzyme or assembly into a functional oligomeric complex. Since proteins are imported into mitochondria in an unfolded

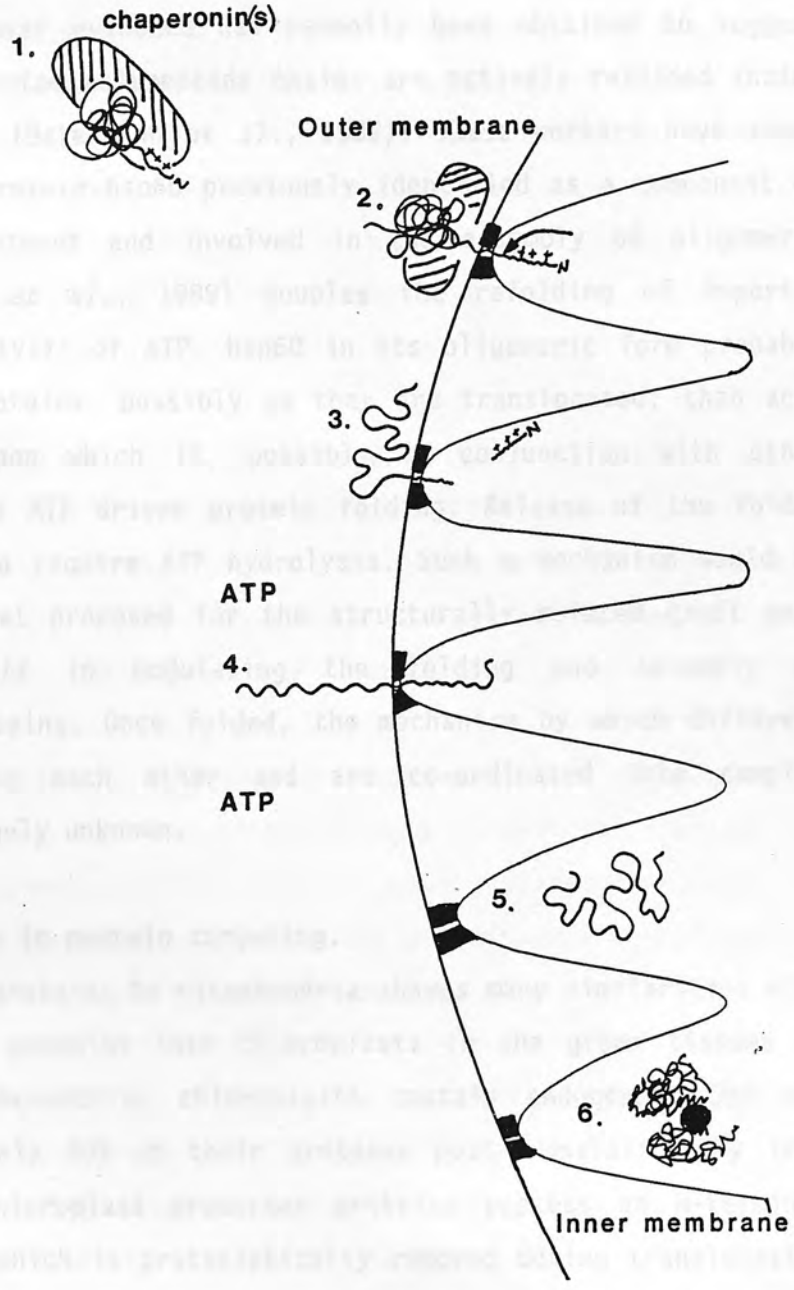

Figure 1.3.
Possible steps accompanying import of a hypothetical protein into the mitochondrial matrix.

(1) Targeting of a precursor to the mitochondrial surface. Chaperonin(s) maintain the import competence of the precursor. (2) Binding of the precursor to the mitochondrial surface, translocation of the presequence across the inner membrane ($\Delta\psi$ dependent) and proteolytic removal of the presequence by the matrix protease. (3) ATP independent partial unfolding of precursor at mitochondrial surface. (4) Complete unfolding and translocation of precursor driven by ATP hydrolysis. (5 and 6) Refolding of the translocated (mature) protein inside the mitochondrial matrix resulting in an active enzyme.

conformation they must therefore refold in order to assume their functional role. Such a refolding process was considered to occur spontaneously however evidence has recently been obtained to suggest that unfolded imported polypeptide chains are actively refolded inside the mitochondrion (Ostermann *et al.*, 1989). These workers have shown that the stress protein hsp60 previously identified as a component of the matrix compartment and involved in the assembly of oligomeric complexes (Cheng *et al.*, 1989) couples the refolding of imported proteins to hydrolysis of ATP. hsp60 in its oligomeric form probably binds unfolded proteins, possibly as they are translocated, then acts as a scaffold upon which it, possibly in conjunction with other proteins, promotes ATP driven protein folding. Release of the folded protein could also require ATP hydrolysis. Such a mechanism would be consistent for that proposed for the structurally related gro*EL* gene product of *E.coli* in modulating the folding and assembly of bacteriophage proteins. Once folded, the mechanism by which different subunits recognise each other and are co-ordinated into complex formation are largely unknown.

1.5. Common themes in protein targeting.

The targeting of proteins to mitochondria shares many similarities with the transport of proteins into chloroplasts in the green tissues of plants. Like mitochondria, chloroplasts contain endogenous DNA but import approximately 80% of their proteins post-translationally from the cytoplasm. Chloroplast precursor proteins possess an N-terminal targeting signal which is proteolytically removed during translocation into the organelle. These transit peptides are similar in length to mitochondrial presequences and similarly contain a high proportion of both hydroxylated and positively charged amino acid residues. Despite the lack of any conserved primary sequence within different chloroplast transit peptides Karlin-Neuman and Tobin (1986) have identified blocks of sequence similarities in several chloroplast precursors, however these have not been observed for all cases studied and their

significance is not clear. As with mitochondrial presequences, the common feature of transit peptides is probably structural.

Several studies have identified putative receptors on the surface of chloroplasts proposed to mediate import of precursors. Cornwell and Keegstra (1987) have used a cross linking approach to identify a 66 kDa protein on the chloroplast surface whereas Pain *et al.*, (1988) using anti-idiotype antibodies identified a 30 kDa protein. Several proteins may be involved in forming precursor recognition sites, possibly for different sub-classes of chloroplast proteins, on the chloroplast surface.

The energetics of chloroplast and mitochondrial transport appear to differ somewhat in that a transmembrane potential is not required for translocation of chloroplast proteins. ATP is required at least in the binding of precursors to the chloroplast surface however recent work has indicated that the major ATP requirement lies across the chloroplast inner membrane (Keegstra, 1989).

Mitochondrial and chloroplast targeting sequences function not only in subcellular targeting but also specify sub-mitochondrial location of the attached polypeptide. A bipartite structure has been proposed for presequences directing proteins to both the mitochondrial intermembrane space and the chloroplast thylakoid membrane (Hartl *et al.*, 1987; Smeekens *et al.*, 1986).

Given the obvious similarity between chloroplast and mitochondrial targeting signals the prospect of mistargeting of proteins in plant cells where mitochondria and chloroplasts co-exist appears possible. Indeed Hurt *et al.* (1986) have shown that a chloroplast transit peptide could direct several different passenger proteins including an authentic chloroplast protein into mitochondria in vitro. These results reflect a low efficiency of targeting and it is unlikely that transit peptides can compete favourably with presequences for mitochondrial import components. This view is supported by the observation that mitochondrial and chloroplast targeting signals directed a bacterial

protein exclusively to the appropriate organelle *in vivo* (Boutry *et al.*, 1987)

The targeting of proteins to the bacterial cytoplasmic membrane and periplasm also involves a post-translational membrane translocation event mediated by an N-terminal targeting signal. These N-terminal signal peptides resemble those of secretory proteins rather than mitochondrial or chloroplast targeting signals in that they consist of a core of hydrophobic amino acid residues with several positively charged residues at the extreme N-terminus and negatively charged residues associated with the junction with the mature protein (Duffaud *et al.*, 1985). Chen and Tai (1985) have shown that while a proton motive force contributed to maximal translocation across the bacterial cytoplasmic membrane, ATP was an absolute requirement. The observation that a relaxed, loosely-folded conformation was required for export of bacterial precursor proteins (Randall and Hardy, 1986) offered a possible role for ATP in common with other translocation systems. Indeed, the requirement of translocated polypeptides to be maintained in an unfolded conformation appears to be one of the fundamental aspects of protein targeting irrespective of the target membrane. Studies with *E.coli* have recently resulted in the purification of three different proteins each of which can maintain precursor proteins in a soluble and thus translocation competent conformation. These chaperonins probably perform a function similar to that of hsp70 class proteins in yeast suggesting that there may be other proteins with a similar function yet to be identified in eukaryotes. Even secretory proteins, which for many years were considered unique in their dependence on a co-translational translocation mechanism, are now recognised as being capable of post-translational translocation indicating that a common mechanism of protein translocation may exist in all cells with differences in targeting sequences and accessory proteins reflecting the specificity of targeting within cells.

CHAPTER 2.

METHODS AND MATERIALS.

2.1. STRAINS AND PLASMIDS

The strains of *Saccharomyces cerevisiae* used in this study are listed in table 2.1 . GR20 was derived from SF747-19D by one step gene disruption at the *CYB2* locus, encoding the cytochrome b_2 precursor protein, (Reid *et al.*, 1988). Strains of *Escherichia coli* used are listed in table 2.2.

Plasmid pGR312 (provided by G. A. Reid), contains a 2.4 kb fragment of genomic DNA from *Saccharomyces cerevisiae* encoding the cytochrome b_2 precursor protein cloned on an EcoR1 fragment. (Figure 2.1).
Plasmid pMC4.b_2 was derived from pMC4, contained the yeast alcohol dehydrogenase promoter (ADH1) and the *CYB2* gene and was provided by G.A. Reid, (Figure 2.2). Plasmid pVT102A was derived from the pVT100-U series (Vernet *et al.*, 1987) and contained a unique EcoR1 restriction site adjacent to the ADH1 promoter, (Figure 2.3).
Plasmid pRC23, designed for high level expression in *E.coli*, (Crowl et al., 1985), was kindly supplied by R. Crowl, Hoffmann La Roche, New Jersey (Figure 2.4). M13.Z1 DNA which contained the 2.3 kb EcoR1/Hind III fragment from pGR312 cloned in M13 mp18, was provided by G.A. Reid.

2.2. CULTURE CONDITIONS AND GROWTH MEDIA

2.2.1. *E.coli*.

All strains of *E.coli* were grown routinely on LB medium, with antibiotic supplementation for maintainence of plasmids. Antibiotic concentration was 50μg/ml for ampicillin and kanamycin, (Maniatis *et al.*, 1982). Cultures were grown at 37°C, (unless otherwise stated), and at 180 r.p.m. on an orbital shaker.
Unless stated otherwise all media formulations are as described in Maniatis *et al.* (1982).

LB-Medium	per litre:	Bactotryptone	10g
		NaCl	5g
		Yeast Extract	5g

Strain	Genotype	Source
SF747-19D	*MAT α, his4, ura3-52, leu2-3, leu2-12*	R.Schekman[b]
GR20	*MAT α, his4, ura3-52, leu2-3, leu2-* 112 *cyb2::LEU2*	G.A.Reid[a]

a) Department of Microbiology, University of Edinburgh.

b) Department of Biochemistry, University of California, Berkeley.

Table 2.1 Yeast strains used with corresponding genotypes.

Strain	Genotype	Source
HB101	*leuB, F⁻, hsd R⁻, hsdM⁻, recA13, ara-14, proA2, xyl-5 lacY1, galK2, RPSL20(Sm^R), mtl-1, SupE44, -*	G.A.Reid
MM294	*Pro⁻, endoA⁻, thi⁻, hsdR⁻ hsdM⁻*	D.Lilley[a]
TG1	*K12, (lac-pro), SupE, thi, hsdD5/F'traD36, ProA⁺B⁺, lacI^q, lacZ M15*	Amersham[b]
POP2136	*H1, trp, lacZ^am, N^am7, N^am57, cI857, H1*	D.Jamieson[c]

a) D.Lilley, Department of Biochemistry, University of Dundee, Dundee.
b) Supplied with Amersham "oligonucleotide mutagenesis system".
c) D.Jamieson, Department of Molecular Biology, University of Edinburgh, Edinburgh.

Table 2.2 Strains of *E.coli* used with corresponding genome.

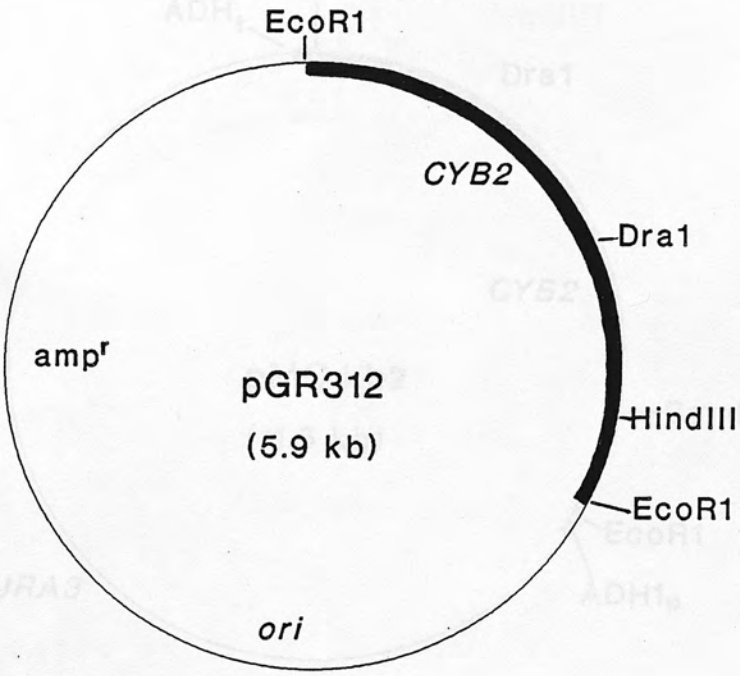

Figure 2.1. Plasmid pGR312.

Construction: A 2.4 kb fragment of yeast genomic DNA isolated in λgt11.b_2.22 (G.A. Reid, unpublished) was transferred to the EcoR1 site of pDS6 (Stueber et al., 1984) to give pGR312.

Comments: The yeast genomic DNA contains the entire *CYB2* gene (which can be isolated as a 1.8 kb EcoR1-HindIII fragment) plus 0.6 kb of 3′ DNA sequence.

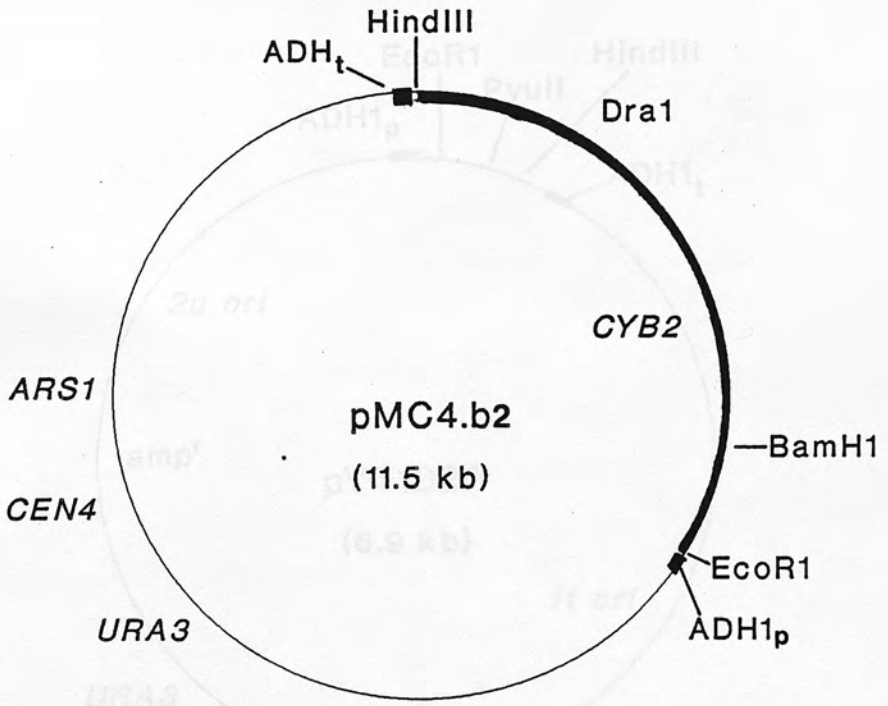

Figure 2.2. Plasmid pMC4.b$_2$.

Construction: The large EcoR1-HindIII fragment from plasmid pMC4 (Hurt et al., 1984), kindly provided by G. Schatz, was ligated to the 2.3 kb EcoR1-HindIII fragment from pGR312 encoding the yeast *CYB2* gene and associated downstream DNA.

Comments: This plasmid permits the expression of DNA fragments in yeast under control of the ADH1 promoter at low copy number.

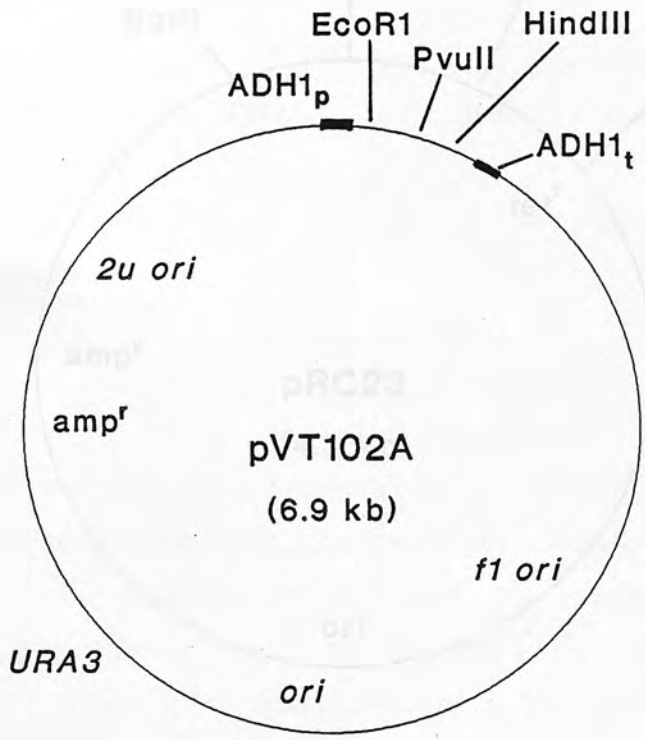

Figure 2.3. Plasmid pVT102-A.

Construction: (a). The EcoR1 site from pVT-U plasmids (Vernet *et al.*, 1986) was removed by filling in ends using Klenow to generate pVT-R1 series.

(b). The pVT-R1 plasmids were cut with BamH1 (in polylinker) ends were filled in with Klenow and EcoR1 linkers were ligated.

(c). Plasmids were cut with EcoR1 and re-ligated to give pVT102-A.

Comments: This vector permits the expression of DNA fragments cloned at the polylinker site under control of the ADH1 promoter on a multi-copy plasmid in yeast. The f1 origin of replication allows packaging of single stranded DNA upon superinfection of E.coli with a filamentous helper phage such as M13K07. These vectors can therefore be used for DNA sequencing and site directed mutagenesis as well as for expression.

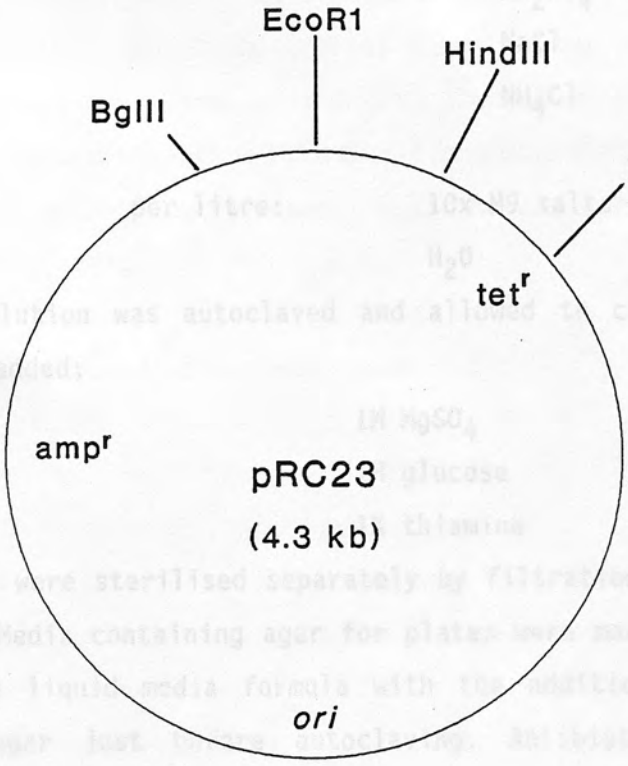

Figure 2.4. Plasmid pRC23.

Construction: See Crowl *et al.* (1985).

Comments: This vector contains a synthetic ribosome binding site (RBS) immediately upstream of the unique EcoR1 cloning site as well as a λP_L promoter, thereby enabling high-level expression of cloned DNA sequences *in vivo* in *E.coli*. Propagation of recombinant plasmids in *E.coli* strains carrying a temperature sensitive λcI857 gene e.g. POP2136, allows the controlled induction of gene expression by shifting cells to 42^0C.

M9 salts (10x).	per litre:	Na_2HPO_4	60g
		KH_2PO_4	30g
		NaCl	5g
		NH_4Cl	10g

M9 Medium.	per litre:	10x M9 salts	100 ml
		H_2O	883 ml

The M9 salt solution was autoclaved and allowed to cool before the following were added;

1M $MgSO_4$	2 ml
1M glucose	10 ml
1% thiamine	1 ml

These solutions were sterilised separately by filtration (thiamine) or by autoclaving.Media containing agar for plates were made according to the appropriate liquid media formula with the addition of 2% (w/v) Oxoid No. 3 agar just before autoclaving. Antibiotic or vitamin supplements were added after autoclaving once the mixture had cooled to approximately 55^{o}C.

2x TY Medium*	per litre:	Bactotryptone	16g
		Yeast Extract	10g
		NaCl	5g
H Plates*	per litre:	Bactotryptone	10g
		Yeast Extract	8g
		Agar	15g
H Top Agar*	per litre:	Bactotryptone	10g
		NaCl	8g
		Agar	8g

* As described in Amersham " Oligonucleotide-directed *in vitro* mutagenesis system " manual.

2.2.2. Yeast

All yeast strains were maintained on YPD plates at 4°C. Cultures were grown at 30°C and 180 r.p.m on an orbital shaker. Long term stocks were made from stationary phase liquid cultures and frozen at -70°C. in 20% (v/v) glycerol. Transformants with specific auxotrophic requirements were grown in minimal medium (glucose), with the appropriate supplement for selection of plasmid.

The following media formulations were used:

YPD.

per litre:	Yeast Extract	10g
	Bactopeptone	20g
	glucose	20g

SD Minimal Medium.

per litre:	Yeast Nitrogen Base*	1.7 g
	$(NH_4)_2SO_4$	5 g
	glucose	20 g

* - Without amino acids or $(NH_4)_2SO_4$

Amino acid supplements were added at 100 mg/l before autoclaving.

2% Lactate Medium.

per litre:	20% Na lactate pH 6.0	100 ml
	Yeast Extract	1.0g
	glucose	3.0g
	KH_2PO_4	1.0g
	NH_4Cl	1.0g
	$CaCl_2.2H_2O$	0.5g
	NaCl	0.5g
	$MgSO_4.7H_2O$	0.7g

For media containing agar for plates, the appropriate liquid media formulation was supplemented with 20% (w/v) Oxoid No. 3 agar before autoclaving and the plates poured after the mixture had reached 55°C.

2.3. SOLUTIONS AND BUFFERS

The following buffers and solutions used in general DNA manipulations were made up in accordance with Maniatis *et al.* (1982).

1. **TE** Buffer: 10 mM Tris.HCl; 1 mM EDTA, pH 7.5

2. **TEG:** 25 mM Tris.HCl; 10 mM EDTA; 50 mM glucose, pH 8.0

3. **Buffers for Restriction Enzyme Digests:**

In most cases 10x restriction enzyme buffers appropriate for each enzyme were supplied by the manufacturer. At all other times buffers were made up in accordance with manufacturers instructions for each particular enzyme.

4. **Buffers for Gel Electrophoresis of DNA.**

10x Loading Buffer: 25% (w/v) Ficoll; 0.25% (w/v) Bromophenol Blue

10x TBE: 0.89 M Tris; 0.89 M boric Acid; 20 mM EDTA pH 8.0

5. **10x DNA Ligase Buffer:** 660 mM Tris.HCl pH 7.6; 50 mM $MgCl_2$; 50 mM DTT; 10 mM ATP.

6. **10x Nick Translation Buffer:** 500 mM Tris.HCl pH 7.2; 100 mM $MgSO_4$; 1 mM DTT; 500 μg/ml BSA.

7. **Buffers for SDS Polyacrylamide Gel Electrophoresis.**

Acrylamide Stock Solution: 30% (w/v) acrylamide;0.8% (w/v) bis acrylamide.

4x Separating Buffer: 1.5 M Tris.HCl pH 8.8; 0.4% (w/v) SDS

4x Stacking Buffer: 0.5 M Tris.HCl pH 6.8; 0.4% (w/v) SDS.

10x Running Buffer: 0.25 M Tris; 2.0 M glycine; 1% (w/v) SDS

2x Sample Buffer: 25% (v/v) 4x Stacking Buffer; 20% (v/v) glycerol; 3% (w/v) SDS; 3% (v/v) 2-mercaptoethanol; 0.01% (w/v) Bromophenol Blue.

Staining solution: 20% (v/v) methanol; 7.5% (v/v) acetic acid; 0.1% (w/v) PAGE Blue 83.

Destaining solution: As above but without PAGE Blue 83.

8. **Western Blot Transfer Buffer:** 250 mM Tris.HCl pH 8.3; 1.5 M glycine.

9. **TBS:** 10 mM Tris.HCl pH 7.5; 150 mM NaCl.

10. **Cytochrome b_2 assay buffers:**

(Cytochrome c reduction); 50 mM KP_i pH 7.4; 0.5 mM EDTA. (If samples of mitochondria were being assayed, assay buffer contained 0.6 M mannitol to maintain their structural integrity.)

(Ferricyanide reduction); 100 mM Na/K P_i pH 7.0; 1 mM EDTA; 100 mM K ferricyanide.

11.. **Solutions for DNA Sequencing.**

Annealing Buffer: 0.1 M Tris.HCl pH 8.0; 50 mM $MgCl_2$.

Termination Mixes:

	T	C	G	A
0.5 mM dTTP	2.5	50	50	35
0.5 mM dCTP	50	2.5	50	35
0.5 mM dGTP	50	50	2.5	35
3.3 mM ddTTP	102.5			
0.1 mM ddCTP		102.5		
0.2 mM ddGTP			102.5	
0.1 mM ddATP				17.5
10 mM Tris pH8				87.5
µl	205	205	205	210

Chase Mix: 0.5 mM each of dNTP, (dCTP, dGTP, dTTP, dATP).

Formamide/Dye mix: 100 ml formamide was deionised by stirring gently with 5 g Amberlite MB1 for 30 min. and filtered. To this was added 30 mg xylene cyanol FF, 30 mg bromophenol blue and Na_2EDTA to 20 mM. The dye mix was stored at room temperature.

Sequencing gel stocks: Stocks were stored at 4°C and warmed to room temperature before preparing the gradient gel.

	Top	Bottom
30% acrylamide:bisacrylamide (29:1)	6 ml	1.25 ml
urea	13.8 g	2.87 g
sucrose	0 g	0.625 g
10x TBE	1.5 ml	1.56 ml
1% (w/v)bromophenol blue	0 ml	0.2 ml
water	30 ml	6.25 ml

12. **Lowry protein determination, solution B:** (Solution A); 2% (w/v) Na_2CO_3; 1.6% (w/v) Na tartrate; 0.4% (w/v) NaOH; 1% (w/v) SDS. Add 1 ml of 4% (w/v) $CuSO_4$ to 100 ml solution A to comprise solution B.

13. **Solutions for site directed mutagenesis:**

Solution A; 200 mM Tris.HCl pH 7.5; 100 mM $MgCl_2$; 10 mM DTT.

Solution B; To 1 μl solution A add the following;

1 μl 100 mM DTT
0.5 μl 20 mM dATP
0.5 μl 20 mM dGTP
0.5 μl 20 mM dCTP
0.5 μl 20 mM dTTP
0.5 μl 20 mM ATP
4.5 μl H_2O
2 units T_4 DNA ligase.

20x SSC; 3 M NaCl; 0.3 M Na_3 citrate.

100x Denhardts; 2% (w/v) BSA; 2% (w/v) Ficoll; 2% (w/v) polyvinylpyrrolidine.

2.4. ISOLATION OF DNA FROM *E.coli*.

Small and large scale isolation of DNA from transformed *E.coli* was performed essentially as described by Birnboim and Doly (1979). CsCl/ethidium bromide buoyant density gradients were carried out according to Maniatis *et al.* (1982).

2.4.1 Small scale preparation of plasmid DNA (Mini-prep).

5 ml cultures of the appropriate transformants was grown to stationary phase at 37°C. Cells were harvested by spinning at 8000 r.p.m. for 5 minutes in a Sorvall SS34 rotor. The pellets were resuspended in 100 μl TEG and the suspension transferred to a clean 1.5 ml Eppendorf tube. Cells were lysed by the addition of 200 μl of 0.2 M NaOH/1% (w/v) SDS, vortexed and left for 5 minutes on ice. 150 μl of 3 M Na acetate was added to precipitate chromosomal DNA and after mixing and incubating on ice for 15 minutes, the suspension was centrifuged for 10 minutes at 4°C in a microfuge. The supernatant was transferred to a clean 1.5 ml Eppendorf tube and plasmid DNA was extracted with 0.5 ml phenol:chloroform:8-hydroxyquinoline, (50:50:0.1), followed by 0.5 ml chloroform, and ethanol precipitated for 20 minutes at -20°C with 0.9 ml absolute ethanol, (see Methods 2.5). The mixture was spun for 10 minutes in a microfuge at 4°C , the supernatant was removed and the pellet dried under vacuum before being resuspended in 50 μl TE.

2.4.2. Large scale preparation of plasmid DNA.

A 1 l culture of the appropriate *E.coli* transformant was grown in LB-medium containing ampicillin to an $O.D._{600}$ = 0.6-0.7. 1.7 ml of 100 mg/ml chloramphenicol was added to amplify plasmid DNA and the cells grown overnight. Cells were harvested at 8000 r.p.m. in a Sorvall SA600 rotor, at room temperature and resuspended in 9 ml TEG. The cell suspension was transferred to two 50 ml Sorval tubes and 0.5 ml 15 mg/ml lysozyme (in TEG) was added to each tube, tubes were then vortexed and left to stand for 15 minutes on ice. 15 ml 0.2M NaOH/1% (w/v) SDS was added to each tube and mixed thoroughly by vortexing. After a further 5 minute incubation on ice, 11.25 ml 3M Na acetate was

added, the tubes were inverted 2-3 times and then left on ice for a further 45 minutes before centrifuging at 16000 r.p.m. for 40 minutes in a Sorvall SS34 rotor at 4°C. The supernatant was transferred to a 300 ml Sorvall tube and the DNA precipitated with 45 ml isopropanol for 5 minutes at room temperature and spun at 10000 r.p.m. in a Sorvall SA600 rotor for 20 minutes at room temperature.

The supernatant was discarded and the pellet drained before being resuspended in a total volume of 5.4 ml T.E. buffer and transferred to a sterile 15 ml corex tube. 5.85 g CsCl was added, dissolved and the mixture left for 1 hour at 4°C to precipitate RNA. This precipitate was removed by spinning at 9000 r.p.m. in a Sorvall SS34 rotor for 30 minutes at 4°C and the supernatant removed into a 10 ml polyallomer ultracentrifuge tube containing 0.6 ml ethidium bromide (10 mg/ml) and mixed. The density of the final solution was adjusted to 1.58-1.60 g/ml and a CsCl/ethidium bromide buoyant density gradient was formed by centrifuging at 35000 r.p.m. in a Sorvall OTD65 ultracentrifuge (r89.5 rotor) for 24-48 hours at 20°C. After centrifuging, plasmid DNA was viewed on the gradient under U.V. light and removed using a 2 ml syringe to puncture the tube wall, into a clean silanized 30 ml corex tube. The ethidium bromide was extracted by shaking 3-4 times with an equal volume of isopropanol saturated with NaCl and water until the top (aqueous) layer was clear. The DNA was then diluted with 8 ml T.E. and precipitated with 8 ml isopropanol for 1 hour at -20°C before being spun down at 8000 r.p.m. in a Sorvall SS34 rotor for 20 minutes at 4°C. The pellet was dried and resuspended in 0.4 ml T.E. and one phenol-chloroform extraction performed before the DNA was precipitated with ethanol. Finally the DNA pellet was vacuum dried and resuspended in 0.2 ml T.E.

The DNA concentration was determined from the absorbance at 260 nm of a sample diluted 200 fold in H_2O against a similar dilution of T.E as a blank (Since an A_{260}=1 is equivalent to a DNA concentration of 20 mg/ml).

2.5. PHENOL / CHLOROFORM EXTRACTION OF DNA

This was performed routinely on DNA samples both during isolation from *E.coli* and between different biochemical manipulations.

The DNA sample was diluted to at least 200 ul as required and the salt concentration adjusted to 0.3 M with 3 M N acetate. An equal volume of phenol:chloroform:8-hydroxyquinoline (50:50:0.1) was added, the mixture vortexed for 10 seconds and spun in a microfuge for 5 minutes. The upper, (aqueous) layer was transferred to a clean 1.5 ml eppendorf tube and an equal volume of chloroform added before vortexing as before. The mixture was microfuged for a few seconds and the upper,(aqueous) layer transferred to a clean 1.5 ml Eppendorf tube. The DNA was then precipitated by the addition of 2 volumes of absolute alcohol (-20°C) and pelleted by spinning for 15 minutes in a microfuge at 4^0C. The pellet was finally dried under vacuum and resuspended in an appropriate amount of T.E.

2.6. RECOMBINANT DNA MANIPULATIONS

All routine DNA manipulations were performed essentially according to Maniatis *et al.* (1982).

2.6.1. Restriction Digestion of DNA

Restriction endonucleases were used according to manufacturers instructions and with suppliers buffers (where provided).

Digests for analytical purposes typically contained 0.1-1 μg DNA in a total volume of 20 μl with 1-3 units of enzyme, 2 μg BSA and 4 μg RNAse A (for mini-prep digests) and were carried out at the appropriate temperature for 1-24 hours. Preparative scale digests for fragment preparation etc contained 5-20 ug DNA with 5-10 units enzyme in volumes between 40-100 μl. When performing double digests, where two enzymes could not be used simultaneously, the DNA was extracted with phenol/chloroform and ethanol precipitated, between each digest.

2.6.2. Electrophoresis of DNA

Restriction digests were assayed by electrophoresis on agarose gels (0.8%-2.0% (w/v)) run in TBE at a constant current of 50 mA. DNA fragments were visualised under ultra violet illumination in the presence of ethidium bromide.

Electroelution of DNA fragments from agarose gels was achieved by excising the desired band of DNA from the gel with a scalpel and using a Biotrap (Schleicher & Schuell Ltd.) to elute the DNA from the band.

2.6.3. Filling in reaction of Klenow fragment.

The large domain or Klenow fragment of *E.coli* DNA polymerase I fills in 5′ overhanging DNA ends in the presence of dNTPs to give blunt ended DNA fragments). The end filling reaction was carried out in a total volume of 30 μl in the presence of the following;

DNA	21.5 μl (diluted as necessary)
10x NTB	3.0 μl
dNTPs	5.0 μl of a 2 mM stock solution (ie each of the four dNTPs = 0.5 mM).
Klenow	2.5 units (0.5 μl of 5 units/μl stock).

The reaction was carried out for 30 minutes at 20°C after which DNA was extracted with phenol:chloroform and ethanol precipitated before further manipulation.

2.6.4. Ligation of DNA fragments

Ligations were carried out in volumes between 10-50 μl, depending on fragment concentration, and in 1x ligation buffer. 1 unit T_4 DNA ligase was used for each reaction. A 3:1 molar ratio of fragment:vector was observed.

2.7. TRANSFORMATION OF *E.coli*.

Performed essentially as described by Mandel and Higa (1970).

Preparation of competent cells.

A culture of the appropriate strain was grown to $O.D._{600}$=0.3 in LB medium at 37°C. Cells were harvested at 8000 r.p.m. at 4°C and resuspended in 0.5 volumes of cold $CaCl_2$. After a 30 minute incubation on ice they were spun down and resuspended in 0.1 volumes of sterile cold $CaCl_2$ and kept on ice ready for use. Competent cells could be stored at -70°C by the addition of glycerol to 20% (v/v) prior to freezing.

Transformation procedure.

200 μl aliquots of competent cells were mixed with plasmid DNA or ligation reaction (1-100 ng in 1-20 μl) and left on ice for 20 minutes. Cells were heat-shocked at 42°C for 2 minutes and 1 ml sterile LB medium was added. Cells were incubated for 1 hour at 37°C, pelleted in a microfuge and resuspended in 100 ul of sterile LB medium before being plated out onto L-agar plates containing 50 ug/ml antibiotic and grown overnight at 37°C, unless otherwise stated.

2.8. TRANSFORMATION OF YEAST USING LITHIUM CHLORIDE

The method of Ito *et al.* (1983) was followed.

A 50 ml culture of the yeast strain to be transformed was grown in YPD medium at 30°C to an $O.D._{600}$=0.4. Cells were harvested by centrifugation at 5000 r.p.m for 5 minutes in sterile corex tubes, washed in 5 ml sterile TE, combined, spun down as before and resuspended in 5 ml sterile TE. Lithium acetate was added to a final concentration of 0.1 M and the cells incubated at 30°C for 1 hour. 200 μl aliquots of cells were removed into sterile Eppendorf tubes and mixed with 5-10 μg of plasmid DNA in 100 μl TE, the mixture was then incubated for 30 minutes at 30°C after which 0.7 ml PEG-4000 was added (giving a final concentration of 35% (w/v)), and the incubation continued for a further 1 hour. The cells were then heat-shocked for 5 minutes at 42°C, pelleted in a microfuge, and washed 3-4 times with sterile water. Finally the cells were resuspended in 100 μl sterile water and plated onto SD+ his minimal medium plates. Transformants took 3-8 days to grow at 30°C and were then picked and streaked onto fresh plates

2.9. DNA SEQUENCING

The dideoxy chain termination method, (Sanger *et al.*, 1977), was used throughout.

2.9.1. Preparation of M13 based single stranded template DNA.

E.coli strain NM522 or TG1 were transformed with M13 RF DNA containing the *cyb2* gene. A colourless plaque was picked using a sterile toothpick and grown for 5-6 hours (or overnight) in 2 ml LB medium at 37°C with good aeration. The culture was transferred to a 1.5 ml Eppendorf tube and spun for 5 minutes in a microfuge. 1.2 ml of the culture supernatant was transferred to an Eppendorf tube containing 0.3 ml 2.5 M NaCl/20% (w/v) PEG 6000. The tube contents were mixed thoroughly and left to stand for 15 minutes at room temperature before spinning for 5 minutes in a microfuge. All of the PEG supernatant was carefully removed with a drawn out pasteur pipette and the phage pellet resuspended in 100 μl TE. 50 μl phenol (saturated with TE), was added, the mixture vortexed for 10 seconds and left to stand for 5 minutes before vortexing again. The mixture was spun for 1 minute in a microfuge, the upper (aqueous) layer was removed to a clean eppendorf tube and extracted twice with 0.5 ml diethyl ether. After removal of all traces of ether, 10 μl 3 M Na acetate pH 5.5 was added followed by 250 μl cold absolute alcohol and the mixture left at -20°C for 1 hour. The DNA was pelleted by spinning in a microfuge for 10 minutes and after drying, was resuspended in 50 μl TE.

2.9.2. Preparation of single stranded template DNA from plasmids containing the f1 origin of replication (phagemids); e.g. pGR401

E.coli strain NM522 or TG1 was transformed with the appropriate plasmid and transformants grown in LB medium containing ampicillin until $O.D._{600}$=0.5 - 0.8. 2 ml of this culture were then infected with M13K07 helper phage at a multiplicity of infection=1 and growth continued for a further hour. 400 μl of this infected culture was then added to 10 ml LB medium containing 50 μg/ml ampicillin and 70 μg/ml kanamycin and the

culture grown overnight with good aeration. Extraction of single stranded DNA then proceeded as for M13 based templates as previously described.

2.9.3. Sequencing of single stranded template DNA.

Anealing of primer to template DNA: 2 μl sequencing primer (2.5 μg/ml) and 1.5 μl 0.1 M Tris.HCl pH 8.0/50 mM $MgCl_2$ were mixed with 7 μl template DNA and sealed in a glass capillary. The capillary was heated to boiling for 3 minutes in a water bath and allowed to cool slowly to room temperature. The neck of the capillary was broken using a diamond pencil and the contents expelled into a 1.5 ml Eppendorf tube.

Sequencing reactions: 2 μl of the annealed primer/template mix were aliquoted into each of four open-topped 1.5 ml Eppendorf tubes. 2 μl of each of the four termination mixes (see Methods 2.3.[10]), were pipetted onto the side of the appropriate tube. 2 μl of a stock solution of Klenow fragment mixed with [^{35}S]-dATP, (10 units Klenow + 40 μCi α [^{35}S]-dATP {600 Ci/mmol}, in a total volume of 90 μl 10 mM Tris.Cl pH 8.0), were pipetted onto the side of each tube and the reaction commenced by spinning the tubes briefly in an Eppendorf 5413 centrifuge. Tubes were incubated at 30°C for 15 minutes after which 2 μl of chase solution (Methods 2.3.[10]) was added to each tube and a second 15 minute incubation at 30°C carried out. 4 μl of formamide/dyes mix (Methods 2.3.[10]) was added to each tube and the samples boiled for 3 minutes to denature the DNA.

Gel electrophoresis of sequencing reactions: After boiling 2-5 μl of sample was loaded on a buffer gradient polyacrylamide gel and run at 30 W for 2 hours. After electrophoresis, the gel was transferred to a piece of dry Whatman 3MM filter paper and excess urea removed by gently running water over the gel surface. The gel was then briefly washed with 10% acetic acid and finally the gel was dried under vaccuum and autoradiographed.

2.10. PREPARATION OF YEAST PROTEIN EXTRACTS

A 50 ml culture of the appropriate yeast strain was grown to an $O.D._{600}$=0.7. A 50 ml centrifuge tube was weighed before the cells were harvested in a Sorvall SS34 rotor, (5000 r.p.m. for 10 minutes), and the weight of the cell pellet calculated. 1 ml of ice cold 10 % (w/v) trichloroacetic acid (TCA), was added for each 0.1 g of cells and the suspension vortexed briefly. Glass beads (0.45-0.50, 40 mesh) were added until just under the surface of the suspension which was then vortexed for 2-4 minutes at top speed. The suspension was re-chilled on ice before being vortexed for a further 2-4 minutes. (N.B. if several samples were being treated, all vortexed suspensions were kept on ice). Liquid was removed from the centrifuge tubes with a pasteur pipette into a 30 ml silanized Corex tube, the glass beads were washed 2-3 times with 10% TCA and the washings combined in the Corex tube. The protein precipitate was spun down at 8000 r.p.m. at 4°C for 5 minutes and as much TCA removed from the pellet as possible before resuspending in 5 ml cold absolute alcohol. The protein precipitate was spun as before and washed twice more with cold absolute alcohol to remove all traces of TCA. The pellet was dried down under vacuum, resuspended in 200-600 μl SDS sample buffer and transferred to a 1.5 ml Eppendorf tube. 1 μl aliquots of 1 M Tris base were added as necessary to give the sample a blue colour. Finally the sample was boiled for 3 minutes and spun for 3 minutes in a microfuge, the supernatant was transferred to a clean Eppendorf before applying to a polyacrylamide gel or storing at -20°C.

2.11. SEPARATION OF PROTEINS BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

The procedure follows that described by Laemmli (1970) with some modifications.

All materials were cleaned before use and glass plates (10 cm x 10 cm) were wiped with absolute alcohol. Plates and spacers were clamped together and sealed with vaseline.

Preparation of separating gel (10%): 10 ml acrylamide stock solution (Methods 2.3.[7]), 12.5 ml distilled water and 7.5 ml 4x separating buffer were mixed in a 100 ml beaker. 300 μl 10% (w/v) APS and 40 μl TEMED were added, quickly mixed and the gel poured between the glass plates to 2-3 cm below the teeth of the comb. The gel solution was overlaid with 2 ml n-butanol (saturated with water) and the gel left to polymerise. After polymerisation, the n-butanol was poured off and the surface of the gel rinsed with water.

Preparation of stacking gel (4.8%): 2.6 ml acrylamide stock solution, 9 ml distilled water and 4 ml 4x stacking buffer were mixed in a 50 ml beaker. 200 μl 10 % (w/v) APS and 20 μl TEMED were added, quickly mixed and poured between the plates to 0.5 cm below the lip of the glass plates. The perspex comb was inserted into the stacking gel, ensuring no bubbles formed around the teeth, and the remaining gel solution added until almost overflowing.

Running the gel: After the stacking gel had polymerised the bottom spacer was removed and the gel clamped to a suitable slab gel electrophoresis tank. 1x running buffer was poured into both resevoirs ensuring the wells were completely submerged, the comb carefully removed from the wells and any residual acrylamide ejected using a syringe. Any air bubbles occupying the bottom space between the plates was also ejected using a syringe. Samples were applied to the wells and electrophoresis carried out at a constant current of 30 mA until the bromophenol blue had reached the bottom. Gels were stained by immersing in staining solution,(Methods 2.3.[7]), heating gently in a microwave oven and leaving for at least 0.5-1 hour. Gels were then transferred to destaining solution, which was changed frequently, until necessary.

2.12. WESTERN BLOTTING

SDS-polyacrylamide gels were run as described above, after electrophoresis gels were not stained and the stacking gel removed. Nylon membrane was cut to fit the size of gel and two sheets of

Whatman 3MM filter paper similarly cut. The nylon membrane and filter paper were soaked in 1x transfer buffer (Methods 2.3.[8]), for at least 5 minutes along with two Scotchbrite pads and a sandwich assembled as follows, ensuring no air bubbles formed between the layers;

Plastic holder (top +ve)
Scotchbrite pad
Filter paper
Nylon membrane
Gel
Filter paper
Scotchbrite pad
Plastic holder (bottom -ve)

The sandwich was placed in an LKB 2005 TRANSPHOR electroblotting unit filled with 5 l of 1x transfer buffer ensuring all wires were covered and the gel is nearest to the -ve terminal. Proteins were transferred for 1-2 hours at 0.6-2.0 Amps or overnight in the cold room at 0.3-0.5 Amps. After transfer, the sandwich was removed from the tank and opened up carefully. The nylon membrane was placed in 100 ml 20% (w/v) skimmed milk powder made up in TBS (Methods 2.2.[9]), overnight with gentle shaking, to block unbound sites

Antibody detection: The blocked membrane was placed in 50 ml 5% milk solution made up in TBS. 10-30 μl antisera was added and the membrane incubated at room temperature with shaking for a further 5 hours. The milk solution was tipped off and the membrane washed for 4x 5 minutes in TBS. 50 ml 5% milk solution containing either, 5 μCi [^{125}I]-Protein A, or 10 μl HRP-conjugated antirabbit antibody (IgG) was added and the membrane incubated as before for a further 2 hours. The membrane was washed as before and if [^{125}I]-Protein A was used, allowed to dry on filter paper and exposed to x-ray film. If HRP-conjugated antirabbit antibody was used, the following solution was made up and added to the membrane;

0.5 ml dianisidine (5 mg/ml)

1.0 ml imidazole (0.1 M, pH7.4)

0.1 ml 30% H_2O_2

8.4 ml dH_2O

The membrane was shaken until a brown colour had developed and the reaction stopped by washing the membrane in distilled water.

2.13. PREPARATION OF YEAST MITOCHONDRIA.

2x 1 l flasks of the appropriate media were inoculated with 1 ml each of a stationary phase starter culture of the appropriate yeast strain and grown to an $O.D._{600}$=0.6 - 0.7. Cells were harvested by spinning in a Sorvall SA 600 rotor at 5000 r.p.m. for 5 minutes and washed once with distilled water. Cells were adjusted to 0.5 g wet weight/ml with Tris.sulphate pH 9.4;10 mM DTT and incubated for 5 minutes at room temperature with shaking. The cell suspension was then spun as above, washed once with 1.2 M sorbitol and spun once again as before. Cells were adjusted to 0.15 g wet weight/ml with 1.2 M sorbitol;20 mM potassium phosphate pH7.4, Zymolase 5000 was added to 5 mg/ml and the suspension incubated at 30°C with gentle shaking for approximately 30 minutes. Formation of spheroplasts was monitored microscopically by examining their capacity to lyse in a hypotonic medium. Spheroplasts were pelleted by spinning at 2500 r.p.m. for 10 minutes and were washed twice with 1.2M sorbitol. The spheroplasts were adjusted to 0.25 g/ml in ice-cold breakage buffer (0.6 M mannitol; 20 mM HEPES.KOH pH 7.4 containing 1 mM PMSF) and broken with 10-15 strokes of a Dounce homogeniser (B), on ice. The homogeniser was rinsed with half the volume of homogenate and the two suspensions combined. The homogenate was spun at 3500 r.p.m. for 5 minutes at 2°C in a Sorvall SS34 rotor and the supernatant saved, (the pellet could be re-homogenised if desired). The supernatant was spun at 9000 r.p.m. for 10 minutes at 2°C in the Sorvall SS34 rotor, the pellet carefully resuspended in approximately 2 ml breakage buffer and the suspension spun at 3500 r.p.m. as before. The supernatant was saved and spun at

9000 r.p.m. as before, the pellet resuspended in a small volume of breakage buffer and again spun at 9000 r.p.m.. Finally the mitochondrial pellet was resuspended in 0.2-1.0 ml breakage buffer and either kept on ice for subsequent manipulation or stored frozen at -20°C.

N.B. the concentration of protein in the final mitochondrial suspension was obtained by measuring the absorbance at 280 nm of a 100-fold dilution in 0.6% SDS, given that an A_{280}=0.2 is equivalent to a protein concentration of approximately 10 mg/ml.

2.14. MITOCHONDRIAL SUBFRACTIONATION.

Mitochondria were prepared as described above, the final suspension was diluted as necessary to a protein concentration of 10 mg/ml with 0.6 M mannitol; 20 mM HEPES pH 7.4..100 μl of the suspension was then diluted 10-fold with 20 mM HEPES pH 7.4; 1 mM TLCK; 30 μM Pepstatin, in a 1.5 ml Eppendorf tube and left for 30 minutes on ice. The suspension was spun in a microfuge for 10 minutes in the cold. The supernatant, containing the intermembrane space (IMS) proteins was removed and kept on ice or frozen at -20°C as required, the pellet (mitoplasts) was resuspended in 100 μl 10 mM Tris.HCl pH 7.4. The mitoplast suspension was frozen rapidly in a dry ice/acetone bath then thawed at 28°C in a water bath. This freeze-thaw cycle was repeated three times to acheive breakage of the mitoplasts. The solution was then spun for 10 minutes in a Sorvall 100.3 rotor at 100000 r.p.m. (4°C). The pellet contained the membrane fraction while the supernatant contained the the matrix fraction

N.B. All buffers contained 1 mM PMSF.

2.15. INDUCTION OF GENE EXPRESSION IN *E.coli* USING pRC23 DERIVED VECTORS.

Cultures of POP2136 harbouring pRC23 derived constructs were grown in LB + ampicillin at 30°C until an $O.D._{600}$=0.3. Cultures were then transferred to a shaking water bath at 42°C and growth continued as

required. If protein extracts for SDS-PAGE and western blotting were required, 1 ml of culture at given time points after induction was removed, spun in a microfuge for 3 minutes, and the pellet resuspended in SDS sample buffer.

2.16. SUBFRACTIONATION OF *E.coli* STRAINS HARBOURING pRC23 DERIVED PLASMIDS.

A 200 ml culture of the appropriate transformant was grown in LB + ampicillin at 30^{0}C until an O.D.$_{600}$=0.3 whereupon gene expression was induced for 90 minutes. Cells were spun down in a Sorvall SA600 rotor at 8000 r.p.m. for 10 minutes and washed twice in 10 mM KP$_i$ pH 7.5. Cells were resuspended in 5 ml 10 mM KP$_i$ pH 7.5. DNAse was added to 50 ug/ml and lysosyme was added to 100 ug/ml before cells were lysed by 2-3 passages through a French pressure cell (5000 p.s.i.). A portion of the lysate was kept as a whole cell sample. The remainder of the lysate was spun at 3000g in a Sorvall SS34 rotor at 4^{0}C. The resultant pellet was resuspended in SDS sample buffer and was kept as an inclusion body sample while the supernatant was spun at 100000g in a Beckman TL100 ultracentrifuge (100.3 rotor). The resultant supernatant was precipitated with 10% TCA and resuspended in SDS sample buffer, this fraction constituted the bacterial cytoplasm. The final pellet, comprising the membrane fraction, was washed 3 times with 10 mM KP$_i$ pH 7.5 and resuspended in SDS sample buffer.

N.B. Inclusion body, membrane and cytoplasm fractions were resuspended in equal volumes of SDS sample buffer. This enabled proportionate amounts of each fraction along with a corresponding amount of the whole cell sample to be compared in western blots and Coomassie Blue stained gels.

2.17. ASSAY OF CYTOCHROME b$_2$ ACTIVITY.

Reduction of cytochrome c:

960 μl assay buffer (Methods 2.3.[11]) was mixed with 20 μl 3 mM cytochrome c solution in a 1 ml perspex cuvette. 5-10 μl of enzyme

containing solution or suspension was added, mixed gently and the absorbance at 549.5nm measured until a steady baseline observed. The assay was commenced by the addition of 10 μl 10 mM L-lactate and mixing

Reduction of ferricyanide:

980 μl assay buffer was added to 5-10 μl of enzyme containing solution or suspension and the absorbance at 420nm measured until a steady baseline observed. The assay was commenced by the addition of 10 μl of 10 mM L-lactate.

2.18. DETERMINATION OF PROTEIN CONCENTRATION

The method of Lowry (1951) was used.

Samples were diluted to a volume of 1 ml as necessary, mixed with 3 ml solution B (Methods 2.3.[12]) and left for 30 minutes. 1 ml of a 1:1 dilution of Folin reagent (in H_2O), was added, mixed and left for a further 30 minutes.

The absorbance at 660nm was measured for each sample.

A series of standard concentrations in the range 0-150 μg/ml BSA was prepared each time an assay was performed and a standard curve of absorbance at 660nm versus protein concentration prepared. The protein concentration in each unknown sample could then be determined from this curve.

2.19. PARTIAL PURIFICATION OF INTERMEDIATE CYTOCHROME b_2.

25 mg of mitochondrial protein (prepared as described in Methods 2.13) were solubilised in 10 mM Tris.HCl pH 7.5; 1 mM EDTA; 200 mM NaCl; 1% Triton X-100. The mixture was stirred at 4^{0}C for 1-2 hours and spun in a Sorvall ultracentrifuge for 1 hour at 105000g. The supernatant was mixed with 1/4 volume acetone and spun at 15000g in a Sorvall SS34 rotor at 4^{0}C. The supernatant was transferred to a clean tube and a second 1/4 volume of acetone added and mixed. The mixture was spun at 40000g for 10 minutes in a Sorvall SS34 rotor at 4^{0}C. The

pellet was drained and resuspended in 3 ml 50 mM KP_i pH 7.0; 200 mM NaCl; 1 mM EDTA; 1mM lactate; 1 mM PMSF and stirred for 1 hour at 4°C. This mixture was then spun at 39000g in a Beckman TL-100 centrifuge at 4°C. The supernatant was discarded and the pellet resuspended in 0.2 ml SDS sample buffer (Methods 2.3.[14]). The presence of intermediate cytochrome b_2 in each fraction was assayed by following the ferricyanide reductase activity (Methods 2.17.)

2.20. 5′ PHOSPHORYLATION OF OLIGONUCLEOTIDES.

(a) For mutagenesis:

The following were mixed in an Eppendorf tube;

200 pmol of oligonucleotide in a total volume of 23 μl

3 μl Tris.HCl pH 8.0

1 μl 0.3 M $MgCl_2$

1.5 μl 0.1 M DTT

1.5 μl 20 mM ATP

5 units T_4 polynucleotide kinase

The mixture was incubated for 45 minutes at 37°C followed by 10 minutes at 68°C to inactivate the enzyme. The phosphorylated oligonucleotide was either used immediately or stored at -20°C.

(b) For screening:

The following were mixed in an Eppendorf tube;

20 pmol oligonucleotide

3 μl Tris.HCl pH 8.0

1.5 μl 0.1 M DTT

1 μl 0.3 M $MgCl_2$

2 μl (20 μCi) [^{32}P]-ATP (3000 Ci/mmol; Amersham PB10168)

H_2O to 30 μl

5 units T_4 polynucleotide kinase

The mixture was incubated and stored as described above.

2.21. SITE DIRECTED MUTAGENESIS PROTOCOLS.

Two methods of introducing site specific mutations within the cytochrome b_2 coding sequence were employed in this study.

A. Double primer method:

Described by Zoller and Smith (1984).

This method uses two oligonucleotides, one designed for mutagenesis, the other forming a perfect match with the template up to a few hundred bases 5′ to the mutagenic oligonucleotide. Extension and ligation from this upstream oligonucleotide will protect the mutagenic oligonucleotide from displacement.

(a) **Annealing:** The following were mixed in an Eppendorf tube;

5 μl ssDNA from a standard miniprep (0.5-1pmol)
1 μl phosphorylated mutagenic oligonucleotide (from 2.18.(a) above).
0.5 μl second oligonucleotide.
1 μl solution A (Methods 2.3.[13]).
H_2O to 10 μl.

The mixture was transferred to a glass cappillary (Supracaps 100 μl), and sealed. Annealing was acheived by boiling for 3 minutes and allowing to cool slowly (30-45 minutes), to room temperature.

Extension and ligation: The annealed DNA was removed into a clean Eppendorf tube. 10 μl freshly prepared solution B (Methods 2.3.[13]), and 2.5 units Klenow fragment were added and the mixture incubated at 15°C for 12-20 hours. On completion of the extension-ligation reaction, a suitable *E.coli* host e.g. TG1 or NM522 was transformed using 1-5 μl of reaction mix.

Screening plaques or colonies using labelled oligonucleotide: The plaques or colonies arising from the above transformation were transferred to nylon (Hybond-N) filters as described in the Amersham booklet "Membrane transfer and detection methods"(1985). Plates were put in fridge for ca. 30 minutes to harden agar. Nylon filters were laid smoothly onto the plate surface and orientation marks made by pushing pins through the filter and into the agar. After 1 minute the

filter was removed and placed colony side up on filter paper soaked in 0.5 M NaOH/1.5 M NaCl for 7 minutes. The filter was placed on dry filter paper to remove excess liquid and then transferred to filter paper soaked in 1.5 M NaCl/0.5 M Tris.HCl pH 7.2/1 mM EDTA for 3 minutes followed by a second, fresh sheet for a further 3 minutes. Finally, the filter was washed briefly in 2xSSC (Methods 2.3.[13]), wrapped in clingfilm and exposed to u.v. radiation for 2-5 minutes on a transilluminator (colony side toward u.v. source), to fix the DNA to the filter.

Hybridization: Prehybridisation was acheived by washing the filter in 6xSSC; 10x Denhardt's; 0.2% (w/v) SDS (Methods 2.3.[13]), for 30 minutes at 65°C in a heat sealed plastic bag. The labelled mutagenic oligonucleotide (from 2.18.(b), above) was then added in 15 ml 6xSSC; 10x Denhardts to the filter which was incubated at 23°C for 60 minutes. The filter was washed briefly in 6x SSC at room temperature and autoradiographed without drying completely. The filter was washed at progressively higher temperatures (5-10°C increments) and autoradiographed after each wash until it was possible to discriminate between wild type and mutant clones. The autoradiographs were then re-aligned with the original plate, mutant colonies or plaques picked and DNA isolated for sequencing.

B. Amersham "Oligonucleotide-directed *in vitro* mutagenesis system":

This system is based on the method described by Nakamaye and Eckstein (1986). The system is outlined below, however a full protocol is provided with the kit.

The mutagenic oligonucleotide is annealed to the single stranded M13 template and extended in the presence of Klenow fragment and T_4 DNA ligase to generate a mutant heteroduplex. The incorporation of a thionucleotide into the mutant strand renders it resistant to cleavage by certain restriction enzymes e.g. NciI. Digestion of the mutant heteroduplex with NciI results in the generation of nicks in the non-mutant strand which allow the use of exonulease III to digest

away all or part of the non-mutant strand. Mutant homoduplex molecules can then be created using the thionucleotide containing (mutant), strand as a template. In addition, on completion of the initial extension/ligation reaction, any remaining single-stranded DNA is removed by a nitrocellulose filtration step thereby increasing the overall efficiency of the system.

2.22. CHEMICALS, ENZYMES AND OTHER MATERIALS.

All chemicals were of analytical grade wherever possible or else general purpose grade and were purchased from BDH Ltd., Poole, Dorset or Sigma Ltd., Poole, Dorset, UK.

Restriction endonucleases and DNA modifying enzymes were purchased from Gibco BRL Ltd., Paisley or Amersham International plc., Amersham, U.K

All radioactive material was purchased from Amersham International plc

Antisera against cytochrome b_2 were raised in rabbits by G.A Reid. HRP-conjugated goat anti-rabbit IgG was of blotting grade and obtained from Bio-Rad Laboratories, Richmond, California, USA.

Zymolase 5000 was purchased from ICN Biochemicals, Cleveland, Ohio.

CHAPTER 3

HIGH-LEVEL EXPRESSION OF THE CYB2 GENE IN YEAST AND *E.coli*.

3.1. Introduction.

Cytoplasmically synthesised, larger molecular weight precursors of mitochondrial polypeptides were first observed by pulse-labelling yeast spheroplasts followed by immunoprecipitation using antibodies directed against mitochondrial proteins (Maccecchini *et al.*, 1979). Precursors detected in this way were observed to disappear rapidly during a chase period reflecting the rapid kinetics of precursor import and processing. Reid and Schatz (1982a) demonstrated that certain mitochondrial precursors could be detected in increasing amounts during growth of a rho$^-$ yeast strain in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) an uncoupler of oxidative phosphorylation. Since mitochondrial import is energy dependent (Nelson and Schatz, 1979) it was possible to uncouple oxidative phosphorylation and hence reduce import of certain precursors without inhibiting long term growth of yeast cells more than approximately 50%.

In particular, the β-subunit of the F_1ATPase precursor showed maximal accumulation in yeast cells grown in the presence of 10 μM CCCP where precursor and mature protein existed in approximately equal amounts. Significantly, this accumulated precursor protein was stable over periods up to several hours and could subsequently be imported into mitochondria after removal of CCCP from the growth medium and processed to its mature size. These observations led Ohta and Schatz (1984) to purify the β-subunit precursor in a denatured form from yeast cells grown in the presence of CCCP. A proportion of this protein could subsequently be renatured such that it could be imported with processing into isolated mitochondria (only in the presence of a yeast cytosolic or reticulocyte lysate fraction). These results demonstrated the feasibility of purifying mitochondrial precursors from yeast in biochemically useful amounts.

Evidence from several laboratories suggests mitochondrial protein import involves receptors located on the surface of the mitochondria, (Gasser *et al.*,1982; Riezman *et al.*, 1983a). It is therefore reasonable to assume that an increased level of synthesis of a mitochondrial

precursor protein could lead to a saturation of import receptors and consequently the accumulation of precursors outside the mitochondria. Gillespie *et al.* (1985) showed that import of the precursor of ornithine carbamyl transferase (pOCT) into isolated mitochondria could be inhibited by addition of larger amounts of a different mitochondrial precursor protein or even by the addition of chemically synthesised mitochondrial presequence peptides. These observations suggested that several different precursors could compete for a common receptor during import and that import was saturable. More recently Pfanner *et al.* (1988a), have proposed that several different receptor-mediated import pathways might converge at a common import site. Saturation of precursor uptake by mitochondria may therefore occur at this stage where all mitochondrial precursors converge.

A multicopy plasmid vector designed for high level expression in yeast cells has been used to express the *CYB2* gene encoding the cytochrome b_2 precursor protein in yeast cells in the experiments described in this chapter. In addition to attempting to accumulate the precursor form by saturating its uptake by mitochondria *in vivo*, an uncoupler of oxidative phosphorylation, CCCP, was used as a means of further reducing the rate of import and thus leading potentially to a greater accumulation of precursor protein.

Expression of a mitochondrial precursor in *E.coli*.

The experiments discussed above, while demonstrating the feasibility of yeast as an organism from which to purify a mitochondrial precursor protein, have not led to the purification of large (ie milligram) amounts of precursors required to conduct biochemical and biophysical experiments. Undoubtedly, a key factor limiting this objective is not that the levels of expression of precursor proteins are low but rather the kinetics of import into mitochondria are rapid preventing large pools of precursors from forming. The expression of a precursor protein in a bacterial system would in principle eliminate the processing of precursors to the mature form caused by their uptake into mitochondria.

In addition, since the efficient expression of eukaryotic genes in bacteria such as *E.coli* is one of the major acheivements of recombinant DNA research, many vectors designed for high level expression are avaialble. Furthermore, the isolation of proteins from yeast is generally a more difficult task than from a bacterial source.

Several workers have attempted to express genes encoding mitochondrial precursors in *E.coli*. Sheffield *et al.* (1986) have cloned the cDNA encoding the rat ornithine carbamyl transferase precursor (pOCT) and expressed it in *E.coli* using a vector containing the *tac* promoter. pOCT accumulates *in vivo* upon induction to approximately 0.1% of total cell protein. Western blots however show the presence of a smaller band corresponding in size to the mature form in approximately equal concentrations. In a similar study, Mattingly *et al.* (1987) expressed the cDNA encoding the rat mitochondrial aspartate aminotransferase precursor (pAAT) which accumulated in *E.coli* progressively up to 8 hours after the induction of gene expression and constituted approximately 1% of total cell protein at maximal levels. pAAT did not appear to be subjected to any significant proteolysis during this accumulation.

These results indicated that at least certain mitochondrial precursor proteins could be stably expressed in *E.coli* without processing to the mature form.

A fusion protein containing the first 22 residues of the presequence from subunit IV of cytochrome oxidase from yeast (COXIV) attached to the N-terminus of mouse DHFR, a cytosolic protein, was expressed in *E.coli* cells by Schatz and Eilers, (1986). This fusion protein was stable and accumulated to approximately 1% of total cell protein. Furthermore the DHFR moiety of the fusion protein retained its enzymic activity despite the fact that the protein aggregated within *E.coli* cells. The above workers were able to purify the COXIV-DHFR fusion protein to homogeneity after its extraction from cells with detergent. This represented the first such purification of milligram quantities of a "mitochondrial precursor protein" and has subsequently allowed the

examination of a number of features associated with the folding and interactions of a precursor during its import into mitochondria (Eilers *et al.*, 1988; Endo and Schatz, 1988). Despite these initial results the availability of alternative precursor proteins would allow the generality of these observations to be examined. Alternatively other mitochondrial precursors may adopt different conformations and participate in different interactions during import particularly since the length and primary structure of different mitochondrial presequences varies considerably. Furthermore, the danger of interpreting experimental data using fusion proteins in the study of mitochondrial import has already been highlighted (Hartl *et al.*, 1986). For these reasons an attempt to express the *CYB2* gene in *E.coli* and subsequently purify the authentic cytochrome b_2 precursor protein in an intact state is further reported in this chapter. In addition the effect of a mitochondrial presequence in *E.coli* is examined by comparing the expression of the corresponding mature protein under the same conditions.

3.2. High level expression of the *CYB2* gene in yeast.

The *CYB2* gene encoding the cytochrome b_2 precursor protein was cloned into vectors designed for high level expression in yeast. Plasmid pMC4.b_2 (Methods fig 2.2) and plasmid pGR401 (figure 3.1) both contain the ADH1 promoter. pMC4.b_2 contains centromere sequences and therefore exists at a few copies per cell whereas pGR401 contains the 2 μ origin of replication and has a copy number around 35 (Erhart and Hollenberg, 1983) thereby facilitating high level expression. The yeast strain GR20 was transformed with each of these plasmids. Transformants were grown on SD + his and on 2% lactate medium and the expression of cytochrome b_2 in transformants was compared with levels in SF747 cells grown on 2% lactate medium, under which conditions the chromosomal *CYB2* gene is specifically induced (Somlo, 1965). The western blot in figure 3.2 shows that cells transformed with pGR401 express most cytochrome b_2

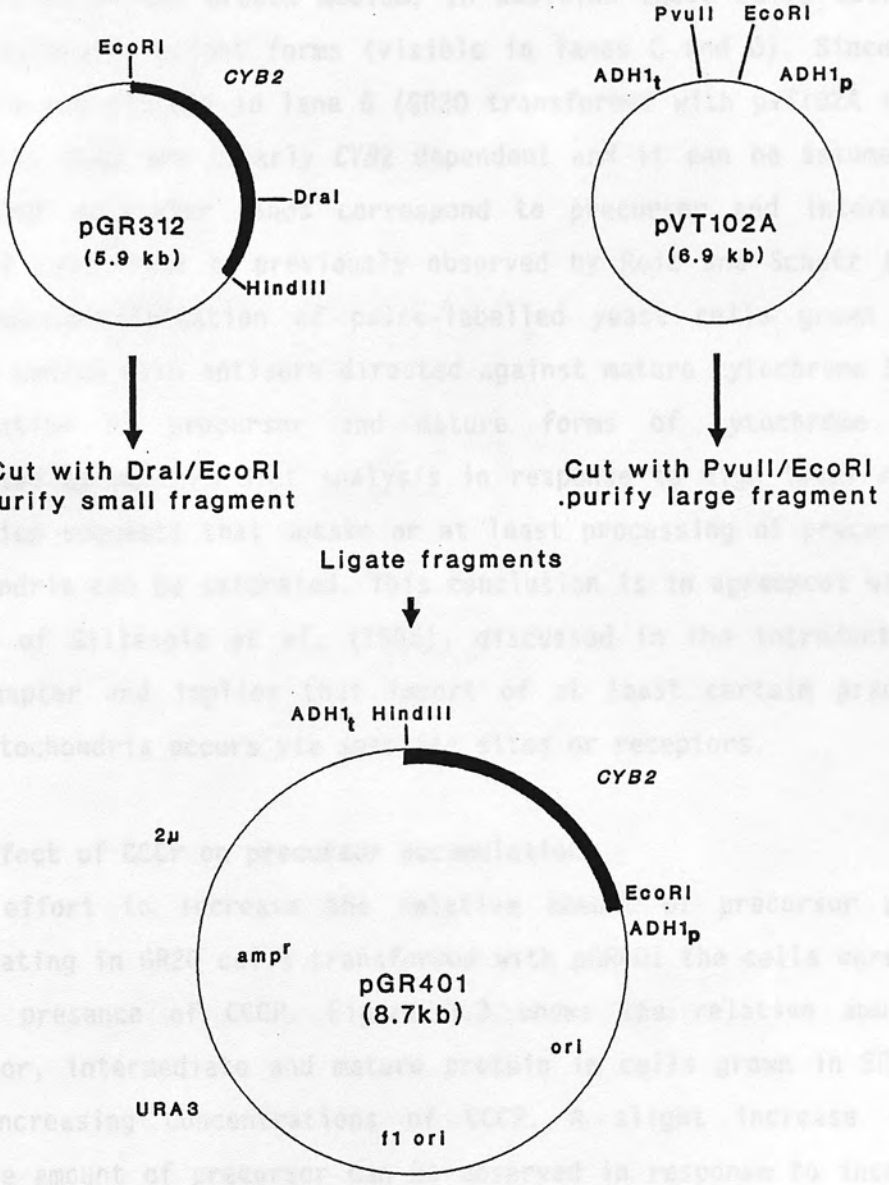

Figure 3.1.

Construction of pGR401.

The 1.8 kb EcoR1/Dra1 fragment from pGR312 containing the entire *CYB2* gene was ligated to the large EcoR1/PvuII fragment of pVT102A to generate pGR401.

irrespective of the growth medium. In addition these cells accumulate higher molecular weight forms (visible in lanes C and D). Since these bands are not visible in lane 6 (GR20 transformed with pVT102A i.e. no *CYB2* gene) they are clearly *CYB2* dependent and it can be assumed that the higher molecular bands correspond to precursor and intermediate forms of cytochrome b_2 previously observed by Reid and Schatz (1982b) upon immunoprecipitation of pulse-labelled yeast cells grown on 2% lactate medium with antisera directed against mature cytochrome b_2. The accumulation of precursor and mature forms of cytochrome b_2 as visualised by western blot analysis in response to high level of *CYB2* expression suggests that uptake or at least processing of precursor by mitochondria can be saturated. This conclusion is in agreement with the results of Gillespie *et al.* (1985), discussed in the introduction to this chapter and implies that import of at least certain precursors into mitochondria occurs via specific sites or receptors.

3.3. Effect of CCCP on precursor accumulation.

In an effort to increase the relative amount of precursor protein accumulating in GR20 cells transformed with pGR401 the cells were grown in the presence of CCCP. Figure 3.3 shows the relative amount of precursor, intermediate and mature protein in cells grown in SD + his with increasing concentrations of CCCP. A slight increase in the relative amount of precursor can be observed in response to increasing concentrations of CCCP. Levels of intermediate and mature cytochrome b_2 are not visibly influenced by increasing concentrations of the uncoupler indicating that only the initial cleavage of the precursor associated with translocation across the mitochondrial inner membrane is affected. The retranslocation of the intermediate form back into the intermembrane space (see chapter 4.1) is apparently unaffected by CCCP. Yeast cells grown in SD + his exhibit a decrease in growth rate related to the concentration of CCCP in the medium (figure 3.4). This observation is in agreement with the results of Reid and Schatz (1982a) who observed a decrease in growth rate of the rho^- yeast mutant

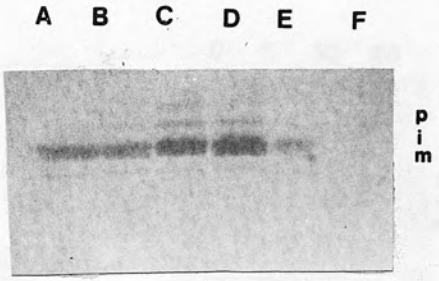

Figure 3.2.

Western blot of total protein from yeast cells expressing cytochrome b_2.

Cells were grown on the appropriate media and proteins extracted in 10% TCA (Methods 2.10.). Approximately 50 µg total protein from each strain/transformant was subjected to SDS-PAGE (10% gel) and proteins transferred to a nylon membrane. Western blots were probed with antiserum directed against mature cytochrome b_2 and visualised using HRP conjugated IgG (Methods 2.12.).

Key: A = GR20(pMC4.b_2) grown on SD+ his.

B = GR20(pMC4.b_2) grown on 2% lactate.

C = GR20(pGR401) grown on SD+ his.

D = GR20(pGR401) grown on 2% lactate.

E = SF747 grown on 2% lactate.

F = GR20(pVT102A) grown on SD+ his.

p = precursor, i = intermediate, m = mature.

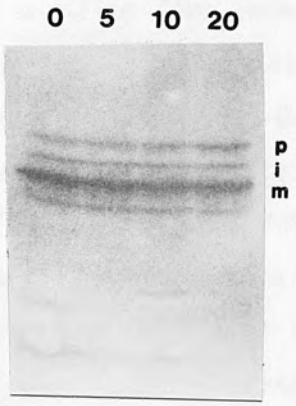

Figure 3.3.

Western blot of total yeast cell protein from strains grown in increasing concentrations of CCCP.

A 200 ml culture of GR20 (+pGR401) was grown in SD + his at 30^{0}C to an O.D.$_{600}$=0.3. The culture was divided into 4 x 50 ml aliquots and CCCP was added to three at final concentrations of 5, 10 and 20 µM. Growth was continued at 30^{0}C for a further 8 hours after which protein was extracted from cells with 10% TCA (Methods 2.10.). Approximately 50 µg total protein from each sample was subjected to SDS-PAGE (10% gel) followed by transfer of proteins to nylon membrane. Western blots were probed with antiserum directed against mature cytochrome b_2 and visualised using HRP conjugated IgG (Methods 2.12).

Key: Values above each lane refer to concentration (µM) of CCCP present in the culture.

p = precursor, i = intermediate and m = mature forms of cytochrome b_2.

D27310B-1 when grown on galactose in response to similar concentrations of CCCP.

3.4. Expression of precursor and mature forms of cytochrome b_2 in *E.coli*

Both the *CYB2* gene and a modified form encoding the mature protein were expressed in *E.coli* using the plasmid vector pRC23 (Methods fig.2.4). This plasmid contains a consensus Shine-Dalgarno sequence located an optimal distance downstream from the tightly regulated λp_L promoter (Crowl *et al.*, 1985). Maintenance of pRC23 based plasmids in *E.coli* strains containing a temperature sensitive λcI857 gene integrated into the *E.coli* chromosome allows the specific induction of gene expression by de-repressing the λp_L promoter as a result of a temperature shift from 30°C to 42°C. Gene products can then be synthesised and accumulated in a relatively short time thereby limiting proteolytic degradation and/or any possible growth inhibitory effects on the cells due to the toxic nature of the polypeptide.

In order to compare the effects of a mitochondrial presequence on expression in *E.coli* and to investigate any potential membrane targeting functions, site directed mutagenesis was used to insert a start codon and restriction site into the *CYB2* gene thereby allowing the expression of mature cytochrome b_2 (figure 3.5). The mutagenesis reaction was carried out using the double primer method (Methods 2.21.(a)). M13.Z1 was used as a source of template DNA. A 26mer oligonucleotide designated 075A (see appendix A) was used to introduce the desired mutation (figure 3.5.) and oligonucleotide B2-1 used to protect the mutagenic oligonucleotide from displacement. Potential mutants were identified using colony hybridisation followed by restriction enzyme digests and DNA sequencing. M13.Z1 carrying the desired mutation was digested with EcoR1 and BamH1, the small fragment isolated and ligated to the large EcoR1-BamH1 fragment from pGR401 to give pGR403. DNA encoding either precursor or mature cytochrome b_2 was isolated from pGR401 and pGR403 respectively on EcoR1-HindIII fragments

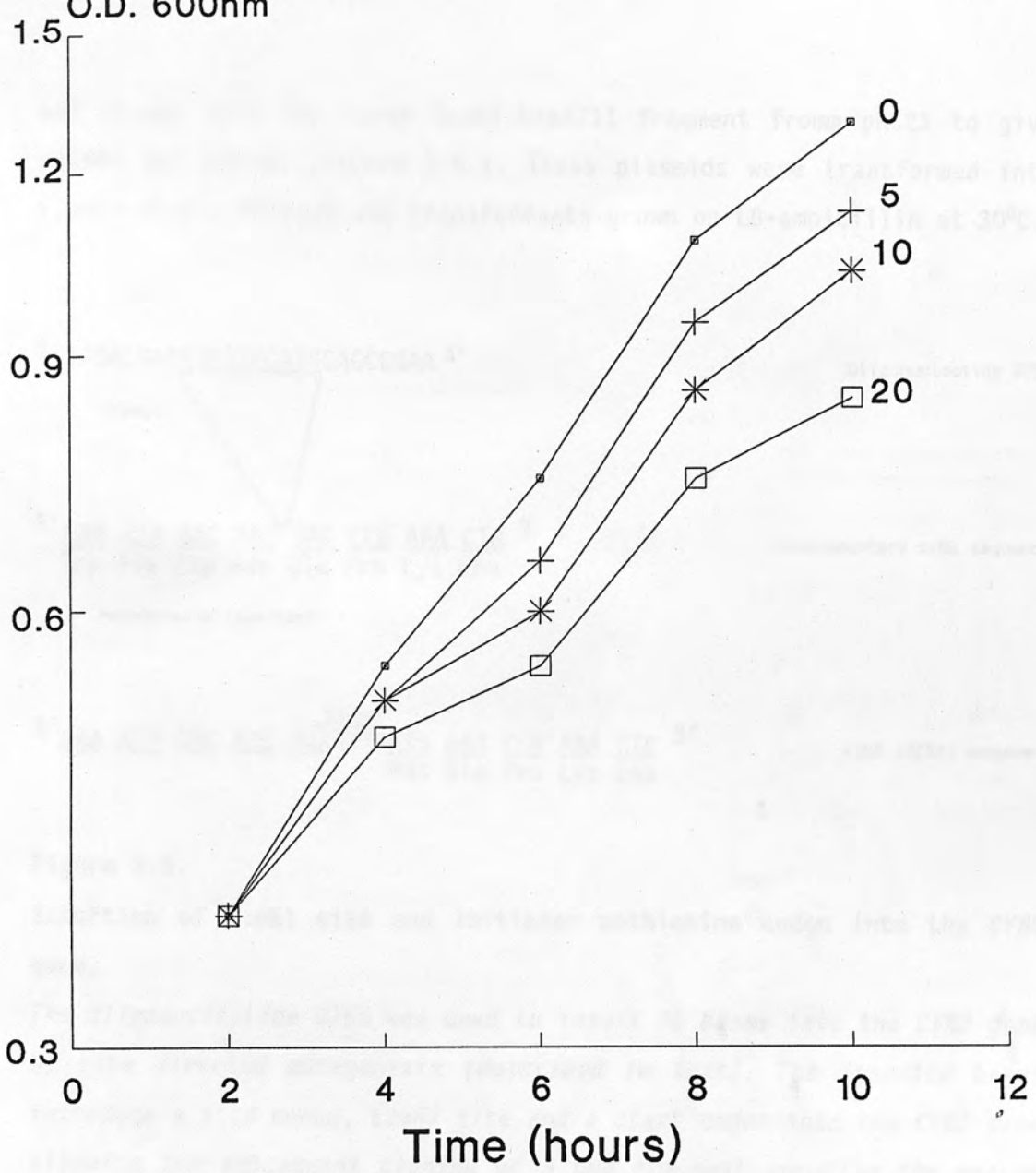

Figure 3.4.

Growth of the yeast strain GR20(pGR401) in the presence of CCCP.

A 200 ml culture of GR20 harbouring pGR401 was grown in SD + his until an $O.D._{600}$=0.3. The culture was divided into four identical aliquots and CCCP was added to a final concentration of 5, 10, and 20 µM in three of the aliquoted cultures. Growth was continued and monitored at the indicated time intervals. Figures assigned to each curve indicate the concentration (µM) of CCCP present.

and cloned into the large EcoR1-HindIII fragment fromm pRC23 to give pRC401 and pRC403 (figure 3.6.). These plasmids were transformed into *E.coli* strain POP2136 and transformants grown on LB+ampicillin at 30°C.

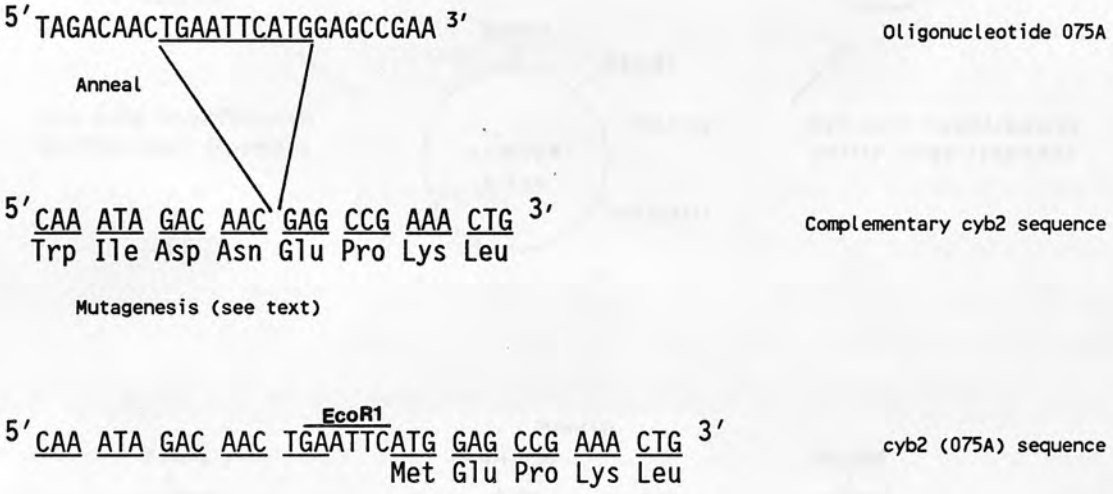

Figure 3.5.

Insertion of EcoR1 site and initiator methionine codon into the *CYB2* gene.

The oligonucleotide 075A was used to insert 10 bases into the CYB2 gene by site directed mutagenesis (described in text). The inserted bases introduce a stop codon, EcoR1 site and a start codon into the CYB2 gene allowing the subsequent cloning of a DNA fragment encoding the mature cytochrome b_2 protein on an EcoR1-HindIII fragment.

N.B. Only that part of the CYB2 gene around the site of insertion is reproduced in the figure.

Transformants were grown and induced as described in Methods (2.15). Total protein extracts prepared at various time points from the above cultures were analysed by SDS-PAGE followed by western blotting as well as staining with Coomassie Blue (figure 3.7 and 3.8). In addition the growth rate of each of the above transformants was measured upon induction of gene expression (figure 3.9).

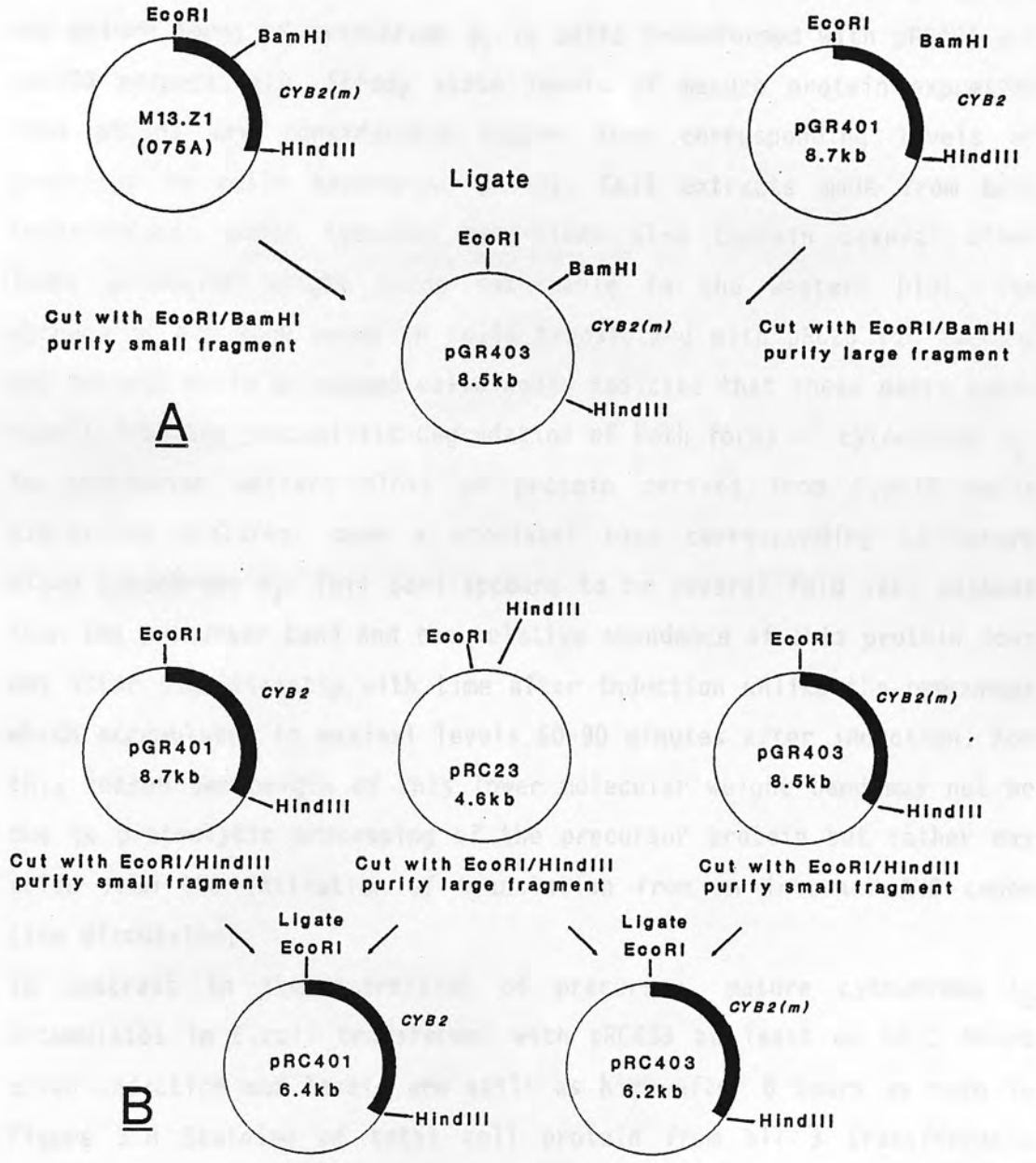

Figure 3.6.

Construction of pRC401 and pRC403.

A. The 550 bp EcoR1-BamH1 fragment from pGR401 was replaced with the 300 bp EcoR1-HindIII fragment from M13.Z1 carrying the mutation constructed using oligonucleotide 075A (see text). The resultant vector was designated pGR403.

B. The EcoR1-HindIII fragments encoding the CYB2 gene and the mutated form encoding the mature cytochrome b_2 protein (CYB2[m]), were inserted into pRC23 to give pRC401 and pRC403 respectively.

The western blot in figure 3.7 shows the accumulation of both precursor and mature forms of cytochrome b_2 in cells transformed with pRC401 and pRC403 respectively. Steady state levels of mature protein expressed from pRC403 are considerably higher than corresponding levels of precursor in cells harbouring pRC401. Cell extracts made from both transformants under inducing conditions also contain several other lower molecular weight bands detectable in the western blot. The absence of any such bands in cells transformed with pRC23 (ie lacking any insert) or in uninduced cells would indicate that these extra bands result from the proteolytic degradation of both forms of cytochrome b_2. In particular western blots of protein derived from *E.coli* cells expressing precursor show a prominent band corresponding to mature sized cytochrome b_2. This band appears to be several fold less intense than the precursor band and the relative abundance of this protein does not alter significantly with time after induction unlike the precursor which accumulates to maximal levels 60-90 minutes after induction. For this reason the origin of this lower molecular weight band may not be due to proteolytic processing of the precursor protein but rather may arise from the initiation of translation from an internal AUG codon (see discussion).

In contrast to the expression of precursor, mature cytochrome b_2 accumulates in *E.coli* transformed with pRC403 at least up to 3 hours after induction and levels are still as high after 6 hours as seen in figure 3.8 Staining of total cell protein from all 3 transformants indicates the relative abundance of precursor and mature cytochrome b_2 in *E.coli* cells. Analysis of Coomassie Blue stained gels by densitometry (using a Shimadzu dual wavelength chromatoscanner) reveals that precursor accumulates up to a maximum of approximately 3% of total cell protein while mature protein constitutes approximately 40% at maximal levels. However, since different proteins stain with varying intensities these values do not necessarily reflect accurately the actual relative amounts of each protein.

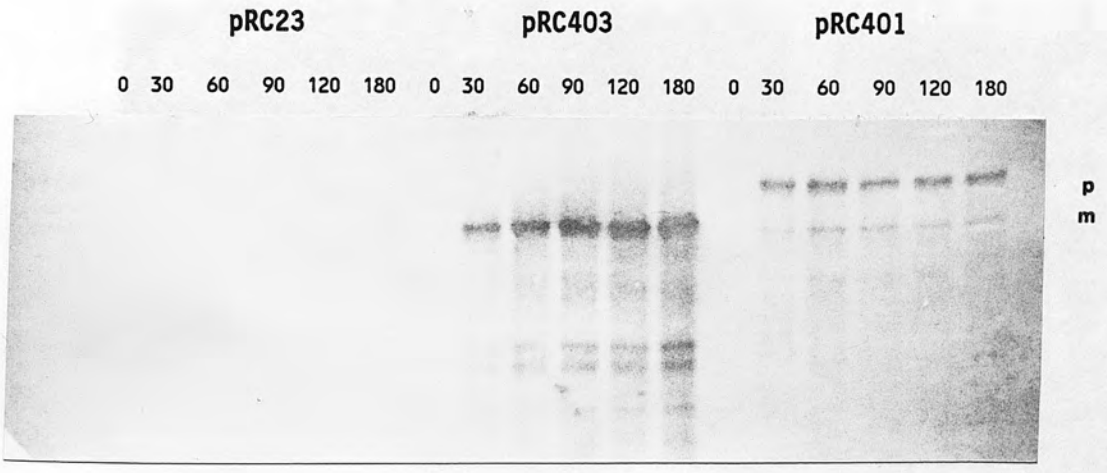

Figure 3.7.

Western blot of *E.coli* cell protein from cells expressing precursor (pb_2) and mature (mb_2) cytochrome b_2.

POP2136 cells transformed with pRC23, pRC403 (mb_2) or pRC401 (pb_2) were induced as described in Methods (2.15.). Total cell protein extracts were made 0, 30, 60, 90, 120, and 180 minutes after induction and approximately 10 µg protein from each extract subjected to SDS-PAGE (10% gel) folowed by transfer to a nylon membrane. Western blots were probed with antiserum directed against mature cytochrome b_2 and developed using HRP conjugated IgG *(Methods 2.12)*

Key: p = precursor, m = mature.

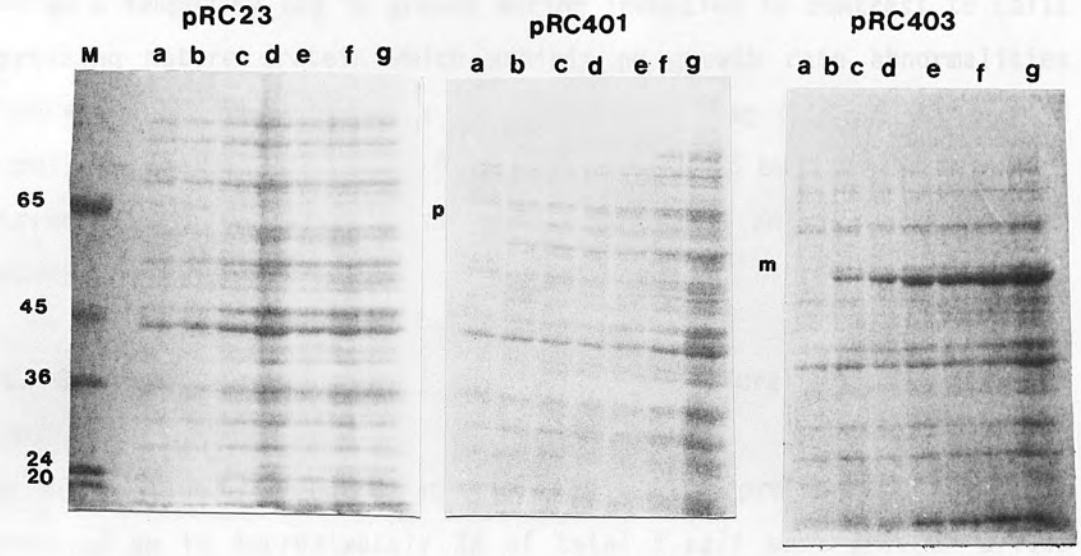

Figure 3.8.

Coomassie Blue staining of *E.coli* protein extracts form cells expressing precursor (pb_2)and mature (mb_2) cytochrome b_2.

The protein samples were prepared according to the legend to figure 3.7. (previous page) with an additional sample prepared from cells 360 minutes after induction of gene expression. Approximately 25 µg protein from each sample was subjected to SDS-PAGE (10% gel) followed by staining with Coomassie Blue.

Key: M = molecular weight markers.

a, b, c, d, e, f and g correspond to samples taken 0, 30, 60, 90, 120, 180 and 360 minutes after induction of gene expression.

Examination of the growth rates of all three transformants after shifting to 42°C reveals that *E.coli* cells expressing precursor protein undergo a temporary lag in growth during induction in contrast to cells expressing mature protein which exhibit no growth rate abnormalities (figure 3.9). The significance of the transient lag in growth exhibited in cells expressing precursor is unclear but could be due to a specific interaction between the precursor protein and an essential *E.coli* component (see discussion).

3.5. Subcellular location of precursor and mature cytochrome b_2 in *E.coli*.

The observation that the cytochrome b_2 precursor protein can be stably expressed up to approximately 3% of total *E.coli* cell protein allows these cells in principle to be used as a source from which the precursor may be purified. Before any such purification the subcellular location of the protein within the cell must be determined. In addition by comparing the localisation of mature and precursor forms any membrane targeting effects conferred by a mitochondrial presequence in *E.coli* can be examined.

E.coli cells were grown and subfractionated as described in Methods (2.16). Figure 3.10 shows the relative distribution of both precursor and mature proteins in each of the subfractions. Figure 3.11 shows the polypeptide profiles in each of the subcellular fractions as detected by Coomassie Blue staining. This allows the purity and degree of cross contamination between the different subfractions to be deduced. Since this appears to be low the western blot in figure 3.10 provides a relatively accurate indication of the subcellular localisation of both proteins. The western blot shows that the majority of both precursor and mature cytochrome b_2 is localised to the inclusion body fraction. Coomassie stained gels (figure 3.11) indicate that this fraction consists almost entirely of cytochrome b_2. In addition a smaller but significant proportion of mature protein is also found in the

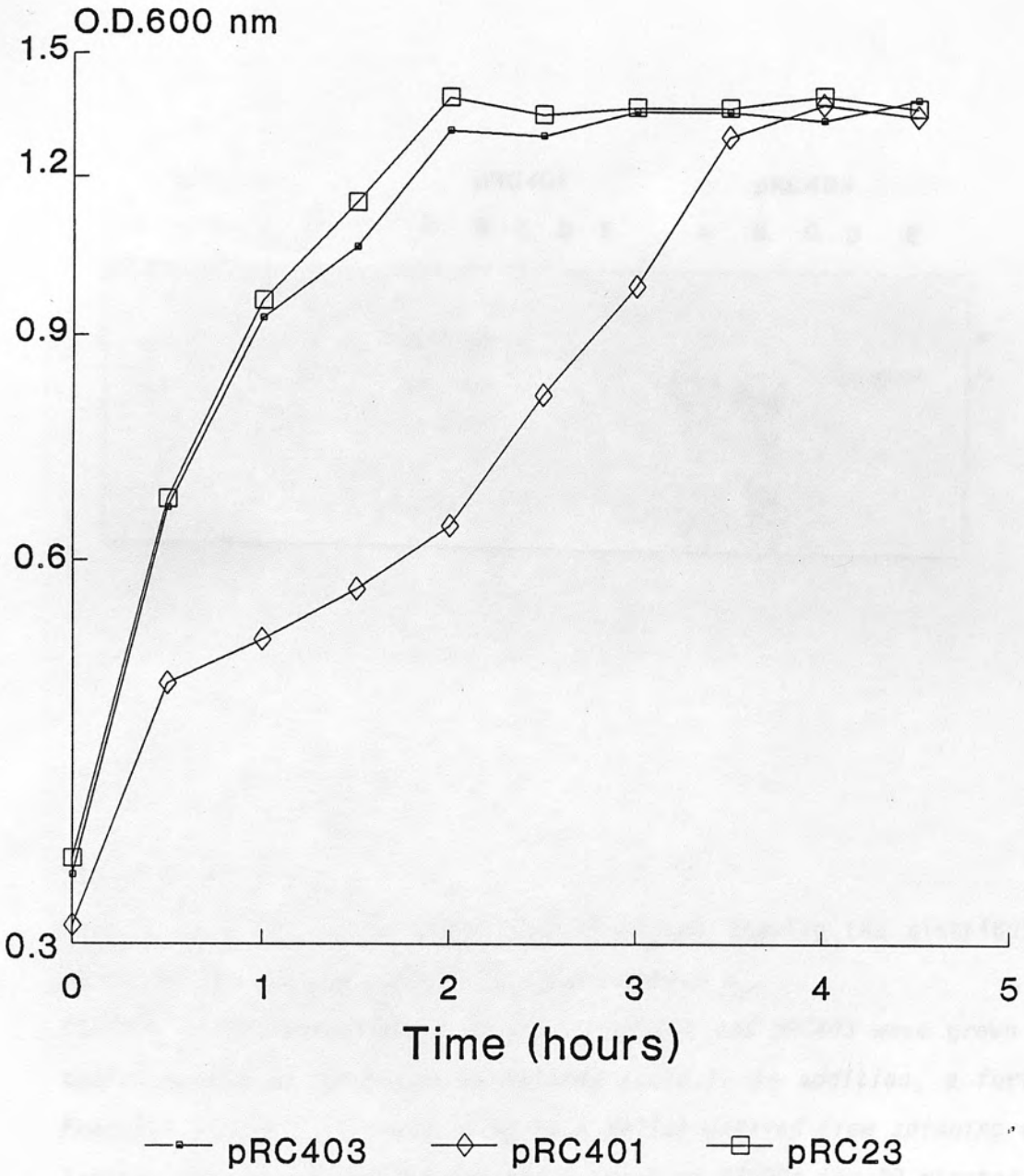

Figure 3.9.

Growth of *E.coli* strain POP2136 expressing precursor and mature cytochrome b_2.

Transformants were grown on LB + ampicillin at 30°C to an $O.D._{600}$ = 0.3. Cultures were then induced as described in methods and growth monitored at the indicated time points by measuring the $O.D._{600}$.

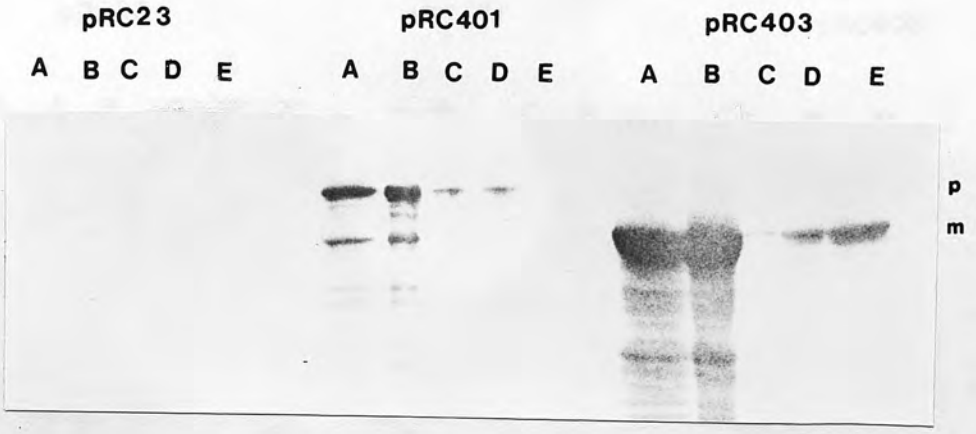

Figure 3.10.

Western blot of *E.coli* subcellular fractions showing the distribution of precursor (pb_2) and mature (mb_2) cytochrome b_2.

POP2136 cells transformed with pRC23, pRC401 and pRC403 were grown and subfractioned as described in Methods (2.16.). In addition, a further fraction (lanes C) corresponding to a pellet derived from spinning cell lysates (after the initial low speed spin) at 17000g for 20 minutes was generated in an attempt to produce purified inclusion body material and reduce contamination of the membrane fraction. Subfractions were resuspended in 400 µl SDS sample buffer and 5 µl of each fraction was subjected to SDS-PAGE (10% gel) followed by transfer of proteins to nylon membrane. Western blots were probed with antiserum directed against mature cytochrome b_2 and visualised using HRP-conjugated IgG (Methods 2.12).

Key: A = Whole cells, B = Low speed spin (inclusion bodies), C = Intermediate speed spin, D = High speed spin (membranes), E = Final supernatant (cytoplasm).

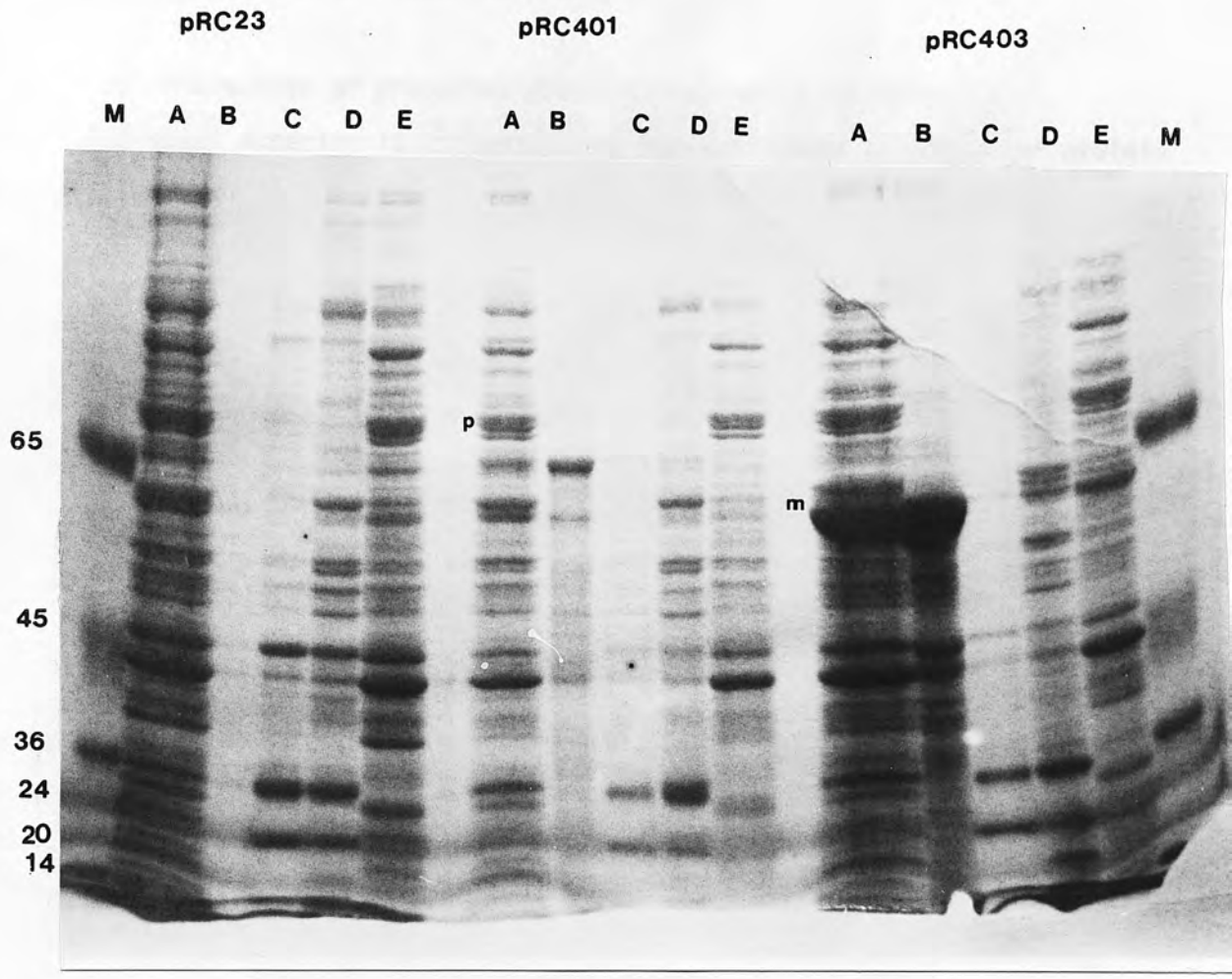

Figure 3.11.

Coomassie Blue staining of *E.coli* subcellular fractions from cells expressing precursor (pb_2) and mature (mb_2) cytochrome b_2.

Subcellular fractions were prepared as described in the legend to figure 3.10. 10 µl of each fraction were subjected to 10% SDS-PAGE and gels stained with Coomassie Blue.

Key: M = Molecular weight markers, A = Whole cells, B = Low speed spin pellet (inclusion bodies), C = Intermediate speed spin pellet, D = High speed spin pellet (membranes), E = Final supernatant, (cytoplasm).

p = precursor, m = mature cytochrome b_2.

cytoplasm. Only a small amount of both precursor and mature protein is associated with the membrane fraction.

3.6. Extraction of precursor protein from inclusion bodies.

The above experiments indicate that the cytochrome b_2 precursor protein can be synthesised in *E.coli* with only limited proteolytic degradation. This lack of significant proteolytic degredation may be due in part to the fact that the protein forms insoluble aggregates which may confer resistance to intracellular proteases, (Stanley and Luzio, 1984). In order to effect the further purification of precursor protein from the inclusion body fraction the solubilisation of this fraction was required.

Purified inclusion bodies were resuspended in 10 mM Tris.HCl; 5 mM EDTA; 200 mM KCl; 1% Triton X-100 and the suspension stirred at 4^{0}C for 1 hour. The suspension was then centrifuged at 100000g for 20 minutes and the presence of precursor protein in the resultant pellet or supernatant fraction assayed by western blotting (figure 3.12). Almost all of the precursor protein remained in the pellet indicating its lack of solubility in the presence of the detergent. A similar result was obtained when the inclusion body suspension was sonicated for 4 x 20 seconds at full power with cooling throughout prior to centrifugation.

The inclusion body material could be solubilised in 8 M urea; 25 mM Tris.HCl pH 7.5. Complete dissolution of all the material required extensive mixing using a Gilson pipette and boiling for 5 minutes (or a longer incubation at 70^{0}C). After solubilisation the inclusion body material remained in solution even at 4^{0}C. However, upon dialysis against solutions containing less than around 4 M urea the material precipitated out. The further purification of inclusion body material was attempted on an FPLC mono Q anion exchange column using a NaCl gradient in the presence of 8 M urea to elute bound protein but this gave poor resolution of eluted proteins.

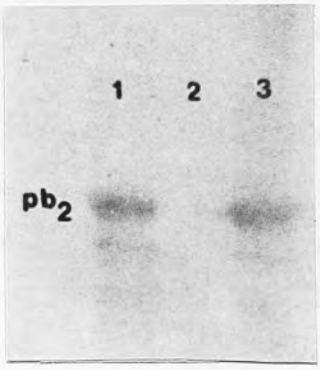

Figure 3.12.

Western blot of inclusion body fractions from E.coli cells expressing precursor cytochrome b_2 after extraction with detergent.

The low speed pellet containing the inclusion body fraction from lysates of E.coli expressing the cytochrome b_2 precursor (prepared as described in the legend to figure 3.10.) was resuspended in 1 ml 10 mM Tris.HCl pH 8.0; 200 mM KCl; 5 mM EDTA; 1% Triton X-100. A portion of this suspension was stirred at 4^0C for approximately 60 minutes and then centrifuged at 100000g for 30 minutes in a Beckman TL100 centrifuge (100.3 rotor). The pellet was resuspended in a volume of SDS sample buffer equal to that of the supernatant and proportionate amounts of supernatant, pellet and original suspension subjected to SDS-PAGE (10% gel) followed by transfer of proteins to a nylon membrane. Western blots were probed with antiserum directed against mature cytochrome b_2 and visualised using HRP-conjugated IgG.

Lane 1 = Whole inclusion body suspension.

Lane 2 = Supernatant.

Lane 3 = Pellet.

3.7. Disscussion.

The evidence for receptor-mediated uptake of mitochondrial precursors in yeast has until recently been circumstantial.The experiments in this study show that at least processing of pre-cytochrome b_2 is saturable *in vivo*. Although the location of this accumulated precursor was not investigated in the experiments described in this chapter, results presented in chapter 4 indicate that the majority of precursor is bound to the mitochondria and some evidence is presented to suggest that the bound precursor is susceptible to added protease. Since processing of precursors is not obligatory for import, these results suggest that the accumulated precursor protein binds to the outer surface of the mitochondria and that its uptake into the organelle is saturable. The presence of CCCP in the growth medium was shown to slightly increase this level of accumulated precursor. In a previous study to investigate the role of CCCP on the uptake of mitochondrial precursors, (Reid and Schatz 1982a) found that no accumulation of precursor b_2 was observed in response to similar concentrations of uncoupler to those used in the experiments described here, however the slight increase observed here may only be detectable above a background of accumulated precursor. The accumulation of other mitochondrial precursors was not examined.

Expression of the full length *CYB2* gene in *E.coli* results in the accumulation of the precursor protein which constitutes up to 3% of total cell protein. When expressed in an identical system however, the mature protein is considerably more efficiently expressed. Why should mature cytochrome b_2 be expressed more efficiently than its corresponding precursor in *E coli*?

Examination of the DNA sequence of plasmids pRC401 and pRC403 between the vector borne Shine-Dalgarno sequence and the initiating AUG codon of each cytochrome b_2 coding sequence indicates a difference in the number of nucleotides separating these features (figure 3.13)

On plasmid pRC401 the Shine-Dalgarno sequence is 9 nucleotides upstream from the initiating ATG codon while on pRC403 the same sequence elements are separated by only 5 nucleotides. A spacing of 7-9

nucleotides has been suggested to be optimal for translational initiation in *E.coli* with shorter sequences more likely to present a barrier to initiation than longer ones (Gold and Stormo, 1987). Expression of the intermediate form of cytochrome b_2 on a pRC23 derived plasmid (see chapter 4) results in a greater accumulation of this form than of the precursor despite identical nucleotide sequence between the Shine-Dalgarno region and the initiator methionine. Thus it is unlikely that this difference alone could account for the disparity in expression levels between precursor and mature forms of cytochrome b_2.

TAAGGAGGAATTCCGGAATG pRC401

Shine-Dalgarno sequence

TAAGGAGGAATTCCGATG pRC403

Figure 3.13. DNA sequence encoding the ribosome binding site on plasmids pRC401 and pRC403.

A more likely explanation is that the precursor is involved in interactions with a cellular component which results in its reduced accumulation. Beck von Bodman *et al.* (1987) expressed both the complete rat hepatic cytochrome b_5 protein and the soluble core domain of the same protein lacking the C-terminal membrane anchor sequence in *E.coli*. While both proteins displayed enzyme activity in the cytoplasmic membrane and cytoplasm respectively, cellular levels of the membrane bound form were approximately only 10% those of the soluble form. More recently, Landry and Bartlett (1989) expressed the small subunit of ribulose-1,5- bisphosphate carboxylase/oxygenase (RUBISCO), a plant chloroplast enzyme, in *E.coli* fused to the carboxyl terminus of staphylococcal protein A′. A fusion between staphylococcal protein A′ and RUBISCO lacking the N-terminal transit peptide was found to be

expressed much more efficiently than a fusion between protein A' and the complete RUBISCO precursor. Moreover, analysis of affinity purified fusion proteins in SDS-PAGE revealed that a 58 kDa protein copurified in approximately equal amounts with fusion protein containing the transit peptide and to a much lesser extent with the fusion lacking this sequence. This 58 kDa protein was shown to be the *E.coli gro*EL gene product on the basis of immunological cross reactivity. Since functional *gro*EL protein is an absolute requirement for *E.coli* viability, not all the available *gro*EL protein was associated with fusion protein. The above authors therefore proposed that the limited availability of *gro*EL protein for binding to fusion protein containing the transit peptide of RUBISCO resulted in lower cellular levels of this protein compared to the fusion lacking this sequence. The association with *gro*EL protein may confer protease resistance to fusions containing the transit peptide.

Owing to the similarity between chloroplast and mitochondrial targeting sequences, the *gro*EL protein would appear a possible candidate for an *E.coli* component limiting the accumulation of the cytochrome b_2 precursor protein. However the association and copurification of a 58 kDa protein (or any other protein), with the inclusion body fraction containing this precursor was not observed. The transient growth lag observed upon expression of pre-cytochrome b_2 may however represent the temporary unavailability of an *E.coli* protein such as that encoded by the *gro*EL gene for essential cellular interactions caused by its association with the precursor. Interestingly, the above authors observed an increase in the total cellular levels of *gro*EL protein in response to expression of fusion protein containing the RUBISCO transit peptide.

Subcellular localisation experiments show that almost all of the precursor and the majority of the mature protein form insoluble aggregates within the *E.coli* cells. This observation is particularly surprising since Black *et al.* (1989) have recently reported the high level expression of fully active, soluble mature cytochrome b_2 in

E.coli. Why then does the mature cytochrome b_2 form an insoluble aggregate in the experiments performed in this study?

Firstly, the expression system used by the above authors utilises a vector designed for *in vitro* mRNA synthesis into which was cloned the complete *CYB2* gene. This construct lacked a defined Shine-Dalgarno sequence upstream from the initiating ATG of the *CYB2* gene but translation of mRNA was initiated at an internal AUG codon corresponding to that encoding the methionine residue at position 6 of the mature protein sequence. A hexanucleotide sequence, $^{5'}$**ACGAGC**$^{3'}$ some 11 bases upstream from this ATG was proposed to act as a Shine-Dalgarno sequence thereby leading to synthesis of "pseudo-mature" cytochrome b_2. The vector chosen for expression of mature cytochrome b_2 in this study however contains a strong consensus Shine-Dalgarno sequence 5 bases upstream of the initiating ATG codon introduced immediately before Glu 1 of the mature protein sequence. Furthermore, initiation of transcription is under control of the λp_L promoter, one of the strongest promoters available for use in *E.coli* which is tightly regulated (Rosenberg *et al.*, 1983). Secondly, expression of "pseudo-mature" cytochrome b_2 is not regulated in the system described above whereas maintenance of pRC403 in a host cell containing a ts λcI857 repressor allows high level expression to be switched on at a defined point. Thus 30 minutes after the induction of gene expression, POP2136 cells transformed with pRC403 have accumulated mature cytochrome b_2 to approximately 12% of total cell protein as measured by densitometric scanning of Coomassie Blue stained gels.

Since the above factors affect the rate of synthesis of cytochrome b_2 in both systems it is possible that a very high level of synthesis of protein could saturate the folding pathway and allow the formation of alternative polypeptide conformations. These conformations could lead to a loss of solubility and aggregation inside the *E.coli*. A smaller but significant fraction of mature cytochrome b_2 is also present in the cytoplasm, while this need not represent correctly folded enzyme, low

levels of cytochrome b_2 activity were detectable in both cell lysates and in the purified cytoplasmic fraction.

In contrast, no precursor protein was ever found in appreciable amounts in the soluble fraction nor was any enzyme activity found in cell lysates. Despite the fact that steady state levels of precursor were several fold lower than those of the mature protein, it cannot be ruled out that aberrant structures do not form during its accumulation in *E.coli*.

Schrank *et al.* (1988) have reported that high level expression of the yeast Mn superoxide dismutase precursor in *E.coli* results in synthesis of an inactive enzyme. Deletion of the mitochondrial presequence by site directed mutagenesis of the *SOD*1 gene however allows synthesis of the fully active protein in *E.coli*. These authors suggest that the mitochondrial presequence prevents the folding of the polypeptide into a functional conformation since no Mn superoxide dismutase activity is observed in petite yeast mutants where the processing step is abolished.

At the present time therefore, the only "mitochondrial precursor" protein shown to exhibit enzyme activity either when purified or within *E.coli* is a COXIV-DHFR hybrid (Eilers and Schatz, 1986). Since DHFR is not a native mitochondrial protein the tight folding of this moiety might enable it to assume its normal conformation in the above fusion protein. Native mitochondrial proteins however may be unable to assume a catalytic conformation and may adopt an import competent state which, in *E.coli* leads to its aggregation and formation of inclusion bodies when expressed at high level. Such aggregation could serve to prevent excessive proteolytic degredation within *E.coli* cells. Recent advances in the purification of proteins in a denatured state and their subsequent renaturation (see chapter 5) might therefore provide a means of obtaining large amounts of precursor proteins from a bacterial source.

CHAPTER 4

TARGETING SIGNALS WITHIN THE CYTOCHROME b_2 PRESEQUENCE

4.1. Introduction.

Nuclear-coded mitochondrial proteins destined for the intermembrane space undergo a more complex sorting and assembly pathway than those precursors destined for the matrix or the matrix face of the inner membrane. Their correct import and processing requires molecular events specific to this sub-group of precursor in addition to those steps common to the import of all mitochondrial precursors. Cytochrome b_2 was first observed to be processed to its mature form via an intermediate molecular weight species after pulse-labeling of intact yeast cells in the presence of CCCP, (Reid and Schatz, 1982b).

This two-step processing could further be visualised by incubating the *in vitro* synthesised precursor with isolated, energised mitochondria at low temperature, (Daum *et al.*, 1982a). Subfractionation of pulse-labelled yeast cells revealed that this intermediate form was localised in the inner mitochondrial membrane. Based on these observations Daum *et al.* (1982a) proposed a model to describe import of cytochrome b_2 and cytochrome c_1. The key element of this model is that the precursor protein is partially translocated across both membranes and as the N-terminal presequence enters the matrix it is proteolytically removed by the chelator-sensitive protease. An intermediate form then remains anchored in the inner membrane until cleavage by an uncharacterised protease releases the protein from its membrane anchor and the mature cytochrome b_2 subunits assemble in the intermembrane space. Cytochrome c_1 was proposed to follow a similar pathway to that describing import of cytochrome b_2 except that processing of the intermediate form to the mature enzyme requires the covalent attachment of a heme prosthetic group prior to proteolytic cleavage and, furthermore, a hydrophobic sequence near the C-terminus keeps the mature cytochrome c_1 anchored to the outer face of the mitochondrial inner membrane.

The deduction of the complete nucleotide sequence of the gene encoding the cytochrome b_2 precursor (Guiard *et al.*, 1985) revealed several interesting structural features within the presequence and enabled a comparison to be made between it and the presequences of two other

intermembrane space proteins cytochrome c_1 and cytochrome c peroxidase. At the extreme N-terminus, all were rich in basic amino acid residues as well as serine and threonine in common with mitochondrial presequences in general. In addition however, these three intermembrane space presequences were unusually long, (80 amino acids in the case of cytochrome b_2) and contained a long stretch of uncharged residues. This last observation lent support to the import model proposed by Daum *et al.* (1982a) since uncharged residues 53-73 within the b_2 presequence could potentially act as a stop-transfer sequence and prevent complete translocation of the presequence into the matrix during import.

Further information on the machinery employed during the import of cytochrome b_2 followed the isolation of a temperature sensitive yeast mutant designated *ts2858* (Pratje and Guiard, 1986). When *ts2858* cells were grown at 36°C, the intermediate form of the protein accumulated. In addition, the same cells also accumulated the uncleaved precursor of the mitochondrially encoded subunit II of cytochrome oxidase (COXII) The above authors noted that the second cleavage site within the cytochrome b_2 precursor, (Asn-Glu) closely resembled the cleavage site on the COXII precursor, (Asn-Asp) and suggested that the mutation in *ts2858* cells was probably within the gene encoding a sequence specific protease, or regulating factor, located in the mitochondrial inner membrane membrane. Interestingly, the precursor form of cytochrome c peroxidase which lacks the sequence Asn-Glu or Asn-Asp is processed normally in *ts2858* cells.

The above authors were also able to confirm the localisation of the intermediate cytochrome b_2 species to the mitochondrial inner membrane and in addition showed that this membrane bound form displayed near normal enzyme activity and had therefore acquired both its heme and flavin prosthetic groups.

A new concept in the import and assembly of intermembrane space proteins was proposed by Hartl *et al.* (1986) after a study of the Rieske Fe/S protein of the ubiquinol:cytochrome c reductase complex of the mitochondrial inner membrane. This protein, located at the outer

face of the mitochondrial inner membrane, is synthesised in the cytoplasm with a 32 residue N-terminal presequence and was found to be completely translocated to the matrix compartment during import. Once inside the matrix, a 24 amino acid peptide was removed from the N-terminal end of the precursor by the matrix protease whereupon an intermediate form with an 8 amino acid extension was retranslocated across the inner membrane to the intermembrane space. This second translocation event was accompanied by the removal of the remaining 8 amino acid extension by an uncharacterised protease in the inner membrane before the polypeptide was assembled into the active bc_1 complex.

In the light of the endosymbiotic theory for the origin of mitochondria, the above authors proposed that complete translocation of the Rieske Fe/S precursor into the mitochondrial matrix delivered the protein to the start of its ancestral assembly pathway. It was further suggested that during evolution, the gene encoding Fe/S protein was transferred from an endosymbiont into the host cell's nucleus, and that the addition of a DNA sequence encoding a positively charged presequence at the N-terminus would then allow the protein to be transported back into the mitochondrial matrix (the cytoplasm of the original endosymbiont) from where it could enter its ancestral assembly pathway. The Rieske Fe/S protein from *Neurospora crassa* and the analogous Fe/S protein from the bacteria, *Rhodopseudomonas sphaeroides* display striking sequence homology (Hauska *et al.*, 1983) as well as comparable structure, topology and function. These observations were further seen to support the idea that the assembly pathway could also be conserved throughout evolution.

Although other workers could readily confirm the results reported by Hartl *et al.* (1986), their model could not account for import of all intermembrane space proteins. van Loon and Schatz (1987) observed that a fusion protein in which the entire presequence of yeast cytochrome c_1 was fused to the N-terminus of the mature portion of subunit IV of cytochrome oxidase, (COXIV) was never found inside the matrix during

import even during careful kinetic analyses unless the uncharged (stop-transfer) sequence in the presequence was deleted.

Hartl *et al.* (1987) then re-examined import of the native, unfused, precursors of cytochrome b_2 and cytochrome c_1 and found that upon inhibition of the matrix protease, in an *in vitro* import assay performed at 8-12^{o}C, the precursor and to a lesser extent the intermediate form were both found to exist in a soluble form inside the mitochondrial matrix. Thus by carefully resolving intermediate stages it was possible to show that cytochrome b_2 and cytochrome c_1 from yeast and the Rieske Fe/S protein from *Neurospora* shared a similar mechanism of import. Hartl *et al.* (1987) further suggested that rather than represent a stop transfer signal, the stretches of non-polar amino acids in the presequences of cytochromes b_2 and c_1 acted as export signals to target the intermediate form of the proteins from the matrix to the intermembrane space. The non-polar presequences of these intermediate forms closely resemble the signal sequences of bacterial plasma membrane proteins and thus may represent the conserved export signal from an endosymbiotic ancestor of mitochondria.

Pfanner *et al.* (1987b) have shown that intermediate cytochrome b_2 can be extracted from mitochondria using 0.1 M sodium carbonate, and therefore is likely to exist in a hydrophilic environment, possibly a proteinaceous channel. This observation further contradicts the idea that a stop transfer signal anchors the intermediate cytochrome b_2 molecule in the lipid bilayer.

The primary aim of the following experimental work was the determination of the N-terminal amino acid sequence of the intermediate form of cytochrome b_2 and thus the identification of the initial cleavage site on the precursor protein. The strategy employed toward achieving this goal follows from the observation of Pratje and Guiard (1986) that the protease responsible for the second proteolytic cleavage during import of the precursor may depend on a specific sequence of amino acids at the site of cleavage, i.e. Asn-Glu/Asp. Alteration of one or both of these amino acid residues might therefore

lead to the abolition of cleavage at this site and a subsequent accumulation of the intermediate form in presumably, the mitochondrial inner membrane. The extraction and purification of this protein would then allow an analysis of the amino acid sequence at its N-terminus. In addition, the precise definition of the intermediate cytochrome b_2 sequence would enable its expression (by deletion of the matrix targeting region from the precursor), in both yeast and *E.coli* allowing a fuller examination of its targeting properties.

4.2. Abolition of the second proteolytic cleavage during import of the cytochrome b_2 precursor in mitochondria *in vivo*.

4.2.1. Site directed mutagenesis of the *CYB2* gene.

Alteration of the amino acid sequence at the second cleavage site was achieved by site-directed mutagenesis of the *CYB2* gene using a synthetic oligonucleotide designated K81 to introduce a single G - A base change in the codon for Glu81 of the precursor protein (i.e. Glu1 of the mature protein sequence) such that this amino acid is replaced by a lysine residue in the mutant polypeptide chain, (figure 4.1)

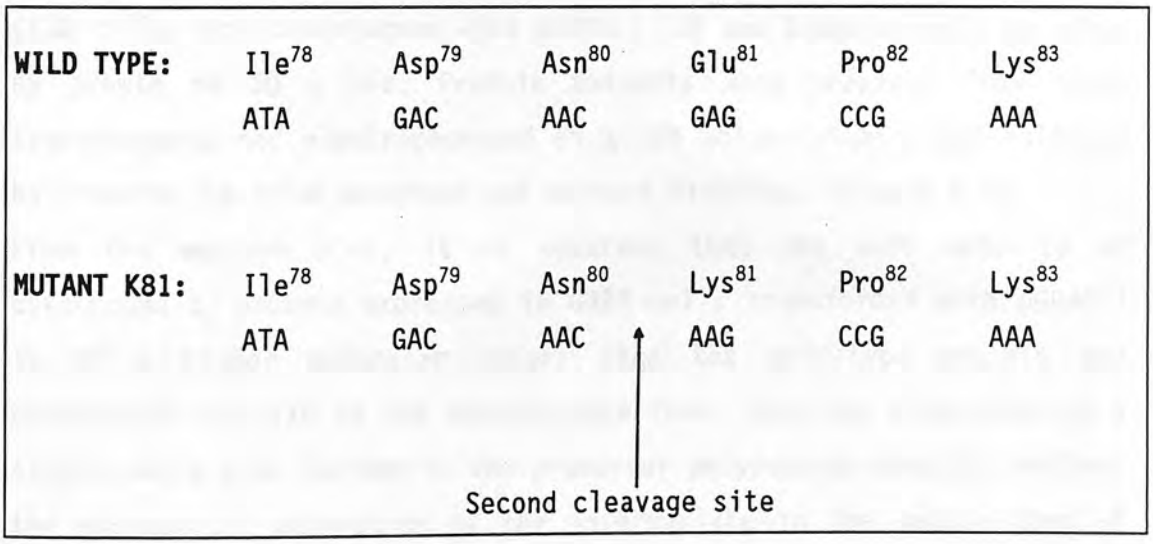

Figure 4.1. (see overleaf for legend)

Comparison of amino acid and nucleotide sequences in wild type and mutant (K81) cytochrome b_2 precursor protein and gene respectively. Superscripts indicate the position of each amino acid residue from the N-terminus of the precursor protein.

The mutagenesis reaction was performed using the Amersham oligonucleotide directed mutagenesis system, (Methods 2.21.(b)). For the complete sequence of oligonucleotide K81 see appendix A.
M13.Z1 RF DNA was transformed into *E.coli* strain TG1 and single-stranded template DNA prepared as described in Methods 2.9.1. On completion of the mutagenic reaction, plaques were screened directly by DNA sequencing.

4.2.2. Expression of the mutant (K81) *CYB2* gene in yeast.
In order to check for the introduction of random mutations within the *CYB2* gene during the mutagenesis reaction, single stranded template DNA identified as containing the desired mutation was sequenced up to the unique BamH1 site within the *CYB2* gene. RF DNA was prepared from such a "clean" clone and the 0.5 kb EcoR1 - BamH1 fragment subcloned into pGR401 cut with EcoR1 and BamH1, (figure 4.2) to generate the vector pGR401i.
GR20 cells were transformed with pGR401i DNA and transformants selected by growth on SD + his. Protein extracts were prepared from these transformants and electrophoresed on a 10% polyacrylamide gel followed by transfer to nylon membrane and western blotting, (figure 4.3)
From the western blot, it is apparent that the vast majority of cytochrome b_2 protein expressed in GR20 cells transformed with pGR401i is of a higher molecular weight than the wild-type protein and corresponds in size to the intermediate form. Thus the alteration of a single amino acid residue in the precursor polypeptide greatly inhibits the proteolytic maturation of the intermediate to the mature form of

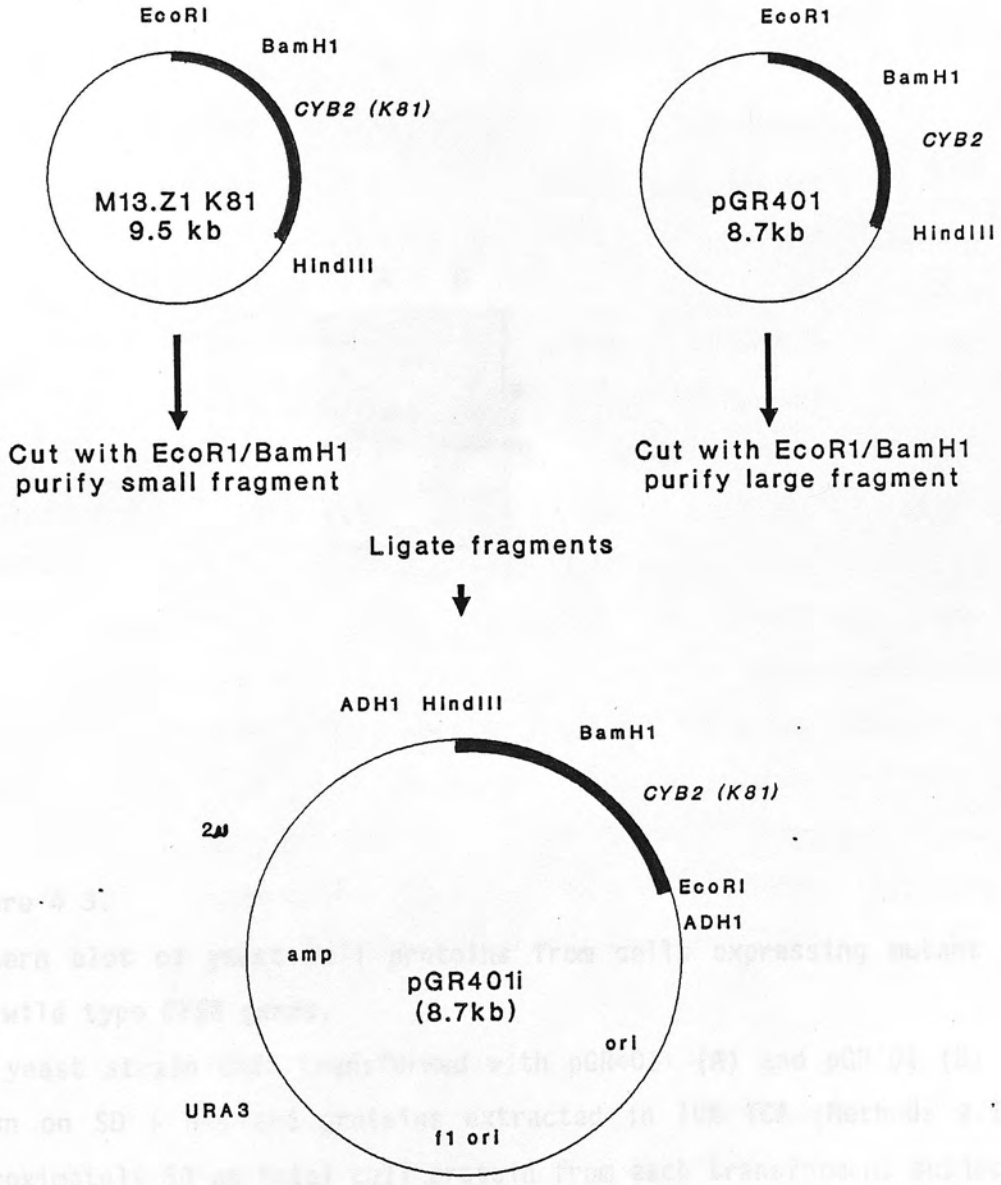

Figure 4.2.

Contruction of pGR401i.

The 450 bp EcoR1/BamH1 fragment from pGR401 was replaced with the same fragment from M13.Z1. carrying the K81 mutation to generate pGR401i.

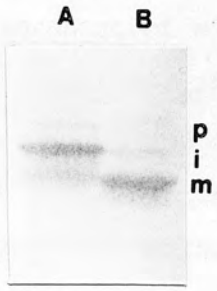

Figure 4.3.

Western blot of yeast cell proteins from cells expressing mutant K81 and wild type *CYB2* genes.

The yeast strain GR20 transformed with pGR401i (A) and pGR401 (B) was grown on SD + his and proteins extracted in 10% TCA (Methods 2.10). Approximately 50 μg total cell protein from each transformant subjected to SDS-PAGE (10% gel) and proteins transferred to a nylon membrane. Western blots were probed with antiserum directed against mature cytochrome b_2 and visualised with HRP-conjugated IgG (Methods 2.12.)

Key: p = precursor, i = intermediate, m = mature.

the protein and results in the accumulation of the intermediate form in yeast cells.

4.3. Submitochondrial location of intermediate cytochrome b_2.

In order to confirm that the protein was accumulating in the mitochondrial inner membrane by abolition of the second cleavage site and not due to events unconnected with its import into mitochondria, mitochondria from yeast cells expressing the K81 mutant and wild-type forms of the *CYB2* gene were isolated and subfractionated. Proteins from each compartment of the organelle were subjected to SDS-PAGE followed by western blotting to determine the location of each cytochrome b_2 species (Figure 4.4)

The figure shows that cells expressing the mutant K81 *CYB2* gene accumulate intermediate cytochrome b_2 in the mitochondrial inner membrane fraction while the wild type cytochrome b_2 is located chiefly in the intermembrane space. This observation is in agreement with those of Daum *et al.* (1982a) and Pratje and Guiard (1986) concerning the location of intermediate cytochrome b_2 and indicates that the K81 mutant protein is on the correct import pathway.

4.4. Catalytic activity of intermediate cytochrome b_2.

The catalytic activity of the intermediate and mature forms of cytochrome b_2 was measured in samples of purified mitochondria. Both cytochrome c reductase and ferricyanide reductase activity was examined, (Methods 2.17.). Specific activities are listed in figure 4.5. Both species of cytochrome b_2 exhibit comparable levels of specific activity when measuring ferricyanide reductase activity but the intermediate form possessed approximately only half the specific activity of the mature protein when cytochrome c was used as the electron acceptor. This result is in contradiction with the earlier work of Pratje and Guiard (1986) who observed that samples of purified mitochondria from wild-type and *ts*2898 cells displayed comparable activities when either electron acceptor was used.

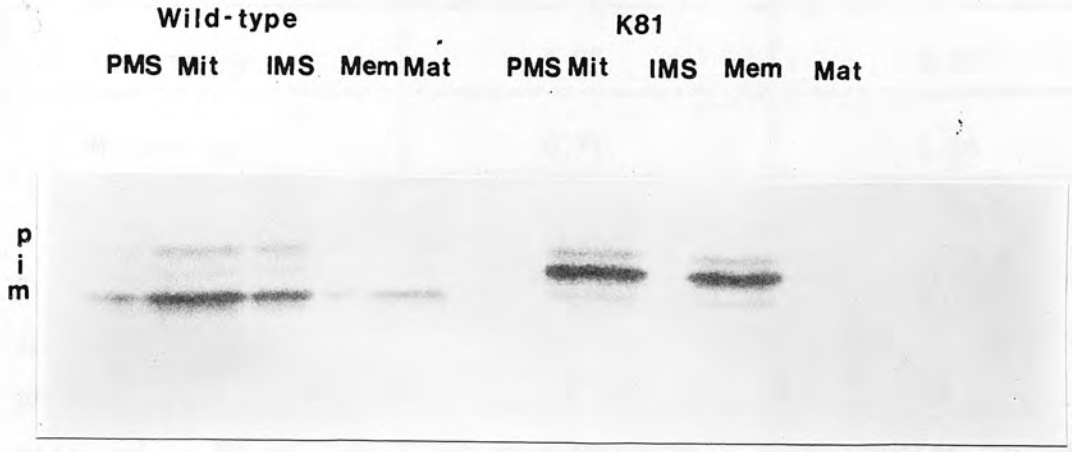

Figure 4.4.

Western blot of submitochondrial fractions from yeast cells expressing mutant K81 and wild-type *CYB2* genes.

Mitochondria were purified from GR20 transformed with pGR401 and pGR401i as described in Methods 2.13 and subfractionated according to Methods 2.14. The concentration of protein in each subfraction was measured (Methods 2.18) and approximately 10 μg of protein from each fraction was subjected to SDS-PAGE (10% gel) and proteins transferred to a nylon membrane. Western blots were probed with antiserum raised against mature cytochrome b_2 and visualised using HRP-conjugated IgG (Methods 2.12.)

Key: PMS = Post Mitochondrial Supernatant.
Mit = Mitochondria.
IMS = InterMembrane Space.
Mem = Membranes
Mat = Matrix.

p = precursor, i = intermediate, m = mature cytochrome b_2.

	cytochrome c reductase	Ferricyanide reductase
Intermediate b_2	0.35	1.35
Mature b_2	0.71	1.36

Figure 4.5. *Cytochrome c and ferricyanide reductase activity of intermediate and mature forms of cytochrome b_2 measured in samples of purified mitochondria. Equal amounts of mitochondrial protein (as measured by the A_{280} of a 100 fold dilution of mitochondrial suspension in 0.6% SDS) were added to each assay. Assays were carried out as described in Methods 2.17. Values given are in µMoles substrate per minute per µg of mitochondrial protein.*

4.5. Determination of the N-terminal amino acid sequence of intermediate cytochrome b_2.

A mitochondrial fraction enriched in intermediate cytochrome b_2 was prepared according to (Methods 2.19). Approximately 100 µg total protein was electrophoresed on a 10% polyacrylamide gel and proteins stained with Coomassie Blue. The identification of the stained band corresponding to intermediate cytochrome b_2 was identified by aligning western blots of a similarly electrophoresed sample. As final confirmation that this band was indeed intermediate cytochrome b_2 it was electroeluted from the gel using a Biotrap (Schleicher and Schuell Ltd.), re-electrophoresed as before and a western blot performed using antisera raised against mature cytochrome b_2. To generate material for amino acid sequence determination approximately 100 µg total protein was electrophoresed on a 10% polyacrylamide gel (N.B. all gel constituents used here were of recrystallised grade) and proteins transferred electrophoretically onto polyvinylidine difluoride (PVDF)

membrane. Bound proteins were then stained with Coomassie Blue and the band corresponding to intermediate cytochrome b_2 was excised using a clean scalpel blade. This segment of membrane was then introduced into the reaction cup of a Waters 743 gas phase automated sequenator and subjected to 8 cycles of Edman degradation. PTH-amino acid derivatives were detected in an on-line analyser by reverse phase HPLC. All steps involved in the final sample preparation and sequencing were carried out by Bryan Dunbar at the Department of Biochemistry, University of Aberdeen. The sequence of the first 8 amino acids is given in figure 4.6. Comparison of the amino acid sequence data obtained above with the amino acid sequence of the complete precursor derived from the DNA sequence (Guiard, 1985) allows the position of the first cleavage of the precursor to be pinpointed. This cleavage takes place between tyrosine 32 and glycine 33 of the precursor protein.

$$Gly^1 - Ser^2 - Thr^3 - Val^4 - Pro^5 - Lys^6 - X - Ser^8$$

Figure 4.6.

Sequence of amino acids at the N-terminus of intermediate cytochrome b_2 as determined by the procedures described in the text. No PTH-amino acid was detected on completion of the 7th cycle of Edman degradation.

4.6. Construction of a mutant form of the *CYB2* gene encoding intermediate sized cytochrome b_2.

The N-terminus of the intermediate cytochrome b_2 polypeptide has been proposed to target this protein from the mitochondrial matrix back across the inner membrane (Hartl *et al.*, 1987). In order to investigate

ATG CTA AAA TAC AAA CCT TTA CTA AAA ATC TCG AAG AAC TGT GAG GCT ATC CTC AGA GCG TCT AAG ACT AGA
Met Leu Lys Tyr Lys Pro Leu Leu Lys Ile Ser Lys Asn Cys Glu Ala Ala Ile Leu Arg Ala Ser Lys Thr

AGA TTG AAC ACA ATC CGC GCG TAC GGT TCT ACC GTT CCA AAG TCC AAG TCG TTC GAA CAA GAC TCA AGA AAA
Arg Leu Asn Thr Ile Arg Ala Tyr Gly Ser Thr Val Pro Lys Ser Lys Ser Pne Glu Gln Asp Ser Arg Lys

CGC ACA CAG TCA TGG ACT GCC TTG AGA GTC GGT GCA ATT CTA GCC GCT ACT AGT TCC GTG GCG TAT CTA AAC
Arg Thr Gln Ser Trp Thr Ala Leu Arg Val Gly Ala Ile Leu Ala Ala Thr Ser Ser Val Ala Tyr Leu Asn

TGG CAT AAT GGC CAA ATA GAC AAC
Trp His Asn Gly Gln Ile Asp Asn Glu Pro Mature protein sequence

A. 80 amino acid N-terminal presequence of the cytochrome b_2 precursor.

ATG GGT TCT ACC GTT CCA AAA TCC AAG TCG TTC GAA CAA GAC TCA AGA AAA CGC ACA CAG TCA TGG ACT GCC
Met Gly Ser Thr Val Pro Lys Ser Lys Ser Pne Glu Gln Asp Ser Arg Lys Arg Thr Gln Ser Trp Thr Ala

TTG AGA GTC GGT GCA ATT CTA GCC GCT ACT AGT TCC GTG GCG TAT CTA AAC TGG CAT AAT GGC CAA GAC AAC
Leu Arg Val Gly Ala Ile Leu Ala Ala Thr Ser Ser Val Ala Tyr Leu Asn Trp His Asn Gly Gln Asp Asn

GAG CCG
Glu Pro Mature protein sequence

B. 48 amino acid N-terminal presequence of intermediate cytochrome b_2

Figure 4.7.

Comparison of N-terminal amino acid sequences from precursor and intermediate forms of cytochrome b_2 with corresponding DNA sequence.

A. The amino acids underlined (Leu2-Tyr32) are those deleted from the precursor by site directed mutagenesis of the CYB2 gene using oligonucleotide 079D.

B. The 48 amino acid presequence present at the N-terminus of the intermediate form created by site directed mutagenesis (above) differs from that produced by processing in vivo only by the presence of a methionine residue at the extreme N-terminus.

the targeting ability of the intermediate presequence, the DNA encoding amino acid residues 2-32 within the presequence was removed from the *CYB2* gene by site directed mutagenesis. A synthetic oligonucleotide designated 079D (see appendix A for sequence) was used to introduce a 93 base pair deletion within the *CYB2* gene. Mutagenesis was carried out using the double primer method (Methods 2.21[a]) and oligonucleotide 102A was used to protect the mutagenic oligonucleotide from displacement during the extension and ligation. Single stranded pGR401 was used as a template in the mutagenesis reaction. Colony hybridisation was used to detect potential mutants while further screening was performed by restriction digests and DNA sequencing. Plasmids carrying the desired mutation can be used to express a form of the *CYB2* gene which encodes the intermediate sized cytochrome b_2 polypeptide and were designated pGR404 (figure 4.7).

4.7. Expression of intermediate sized cytochrome b_2 in yeast.

GR20 cells were transformed with pGR404 and transformants grown on SD + his. Whole cell protein extracts were made from GR20 cells transformed with pGR401, pGR404 and pGR401i. The western blot in figure 4.8 shows that the intermediate cytochrome b_2 produced in cells transformed with pGR404 is not significantly processed to a smaller molecular weight form nor results from cleavage from a higher molecular weight form unlike wild type protein which undergoes the normal two maturation steps. This failure of the protein to undergo processing could in principle be due to its failure to be targeted to the mitochondrion particularly since it lacks amino acids 2-32 of the precursor which have been shown to contain the information nessecary for mitochondrial targeting (R. Pallister, Ph.D. thesis, in preparation). This possibility was investigated by fractionating yeast cells into mitochondrial and cytoplasmic fractions and examining each fraction for the presence of cytochrome b_2. Figure 4.9 compares the cellular distribution of intermediate and mature cytochrome b_2 in yeast cells transformed with pGR404 and pGR401 respectively, as detected by western

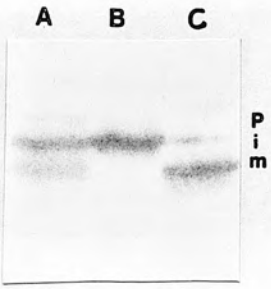

Figure 4.8.

Western blot of yeast cell proteins from cells transformed with pGR401i (A), pGR404 (B) and pGR401 (C).

Proteins were extracted from the above strains grown on SD + his in 10% TCA (Methods 2.10.). Approximately 50 µg of protein from each sample was subjected to SDS-PAGE (10% gel) and proteins transferred to a nylon membrane. Western blots were probed with antiserum raised against mature cytochrome b_2 and visualised using HRP-conjugated IgG (Methods 2.12.)

Key: p = precursor, i = intermediate, m = mature cytochrome b_2.

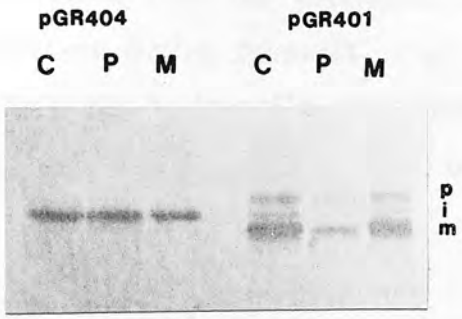

Figure 4.9.

Western blot of subcellular fractions from yeast cells transformed with pGR404 and pGR401.

Mitochondria from both transformants were purified as described in Methods 2.13. except that a fraction of the homogenized spheroplasts were kept to provide a "whole cell" sample. Protein concentrations in each subfraction were determined (Methods 2.18) and proportionate amounts of protein subjected to SDS-PAGE (10% gel). Proteins were transferred to a nylon membrane and western blots were probed with antiserum directed against mature cytochrome b_2 and visualised with HRP-conjugated IgG (Methods 2.12.).*

**Since mitochondria contain approximately 10% of total yeast cell protein, the amount of mitochondrial protein applied to the gel above was approximately 10% that of whole cell and post mitochondrial supernatant samples.*

Key: C = Whole cells, P = Post mitochondrial supernatant, M = Mitochondria.

p = precursor, i = intermediate, m = mature.

blotting. As expected virtually all the mature sized cytochrome b_2 in cells transformed with pGR401 is located in the mitochondria. The amount detected in the cytoplasmic fraction can be attributed to leakage of the protein from the intermembrane space due to disruption of the outer membrane during preparation of mitochondria. In contrast, intermediate cytochrome b_2 in cells transformed with pGR404 is found in both mitochondrial and cytoplasmic fractions in roughly equal proportions.

4.8. Location of intermediate sized cytochrome b_2 associated with mitochondria.

Despite its association with mitochondria, the observed lack of processing of intermediate sized cytochrome b_2 in yeast cells harbouring pGR404 suggests that the protein does not follow the normal import pathway. In order to investigate whether the protein is imported into the mitochondrion or merely bound to the surface of the outer membrane, mitochondria from yeast cells harbouring pGR401 and pGR404 were isolated and incubated with proteinase K, fractions were then examined by western blotting for the presence of either form of cytochrome b_2 (figure 4.10). The blot shows that while the amounts of mature cytochrome b_2 in the presence and absence of protease remain constant, much less of the intermediate-sized protein remains present after protease treatment.

4.9. Expression of intermediate cytochrome b_2 in *E.coli*.

Results presented in chapter three show that high level expression of both precursor and mature forms of cytochrome b in *E.coli* results in the formation of inclusion bodies with mature protein expressed in considerably greater amounts than precursor. In addition although neither protein shows any association with *E.coli* membranes a significant amount of mature protein remains soluble in the cytoplasm. Does the intermediate form behave in a similar way upon its expression in *E.coli*?

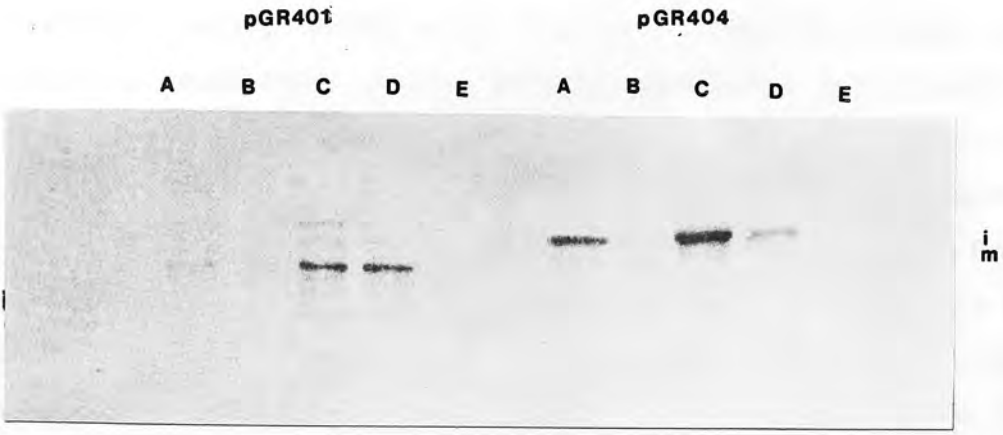

Figure 4.10.

Western blot of mitochondrial and cytoplasmic fractions from yeast cells transformed with pGR401 and pGR404 treated with Proteinase K.

Mitochondria were purified from yeast cells transformed with pGR401 and pGR404 as described in Methods 2.13. and the protein concentration of mitochondrial and post mitochondrial (cytoplasmic) samples determined (Methods 2.19.). Approximately 25 µg of mitochondrial protein and a proportionate amount of protein from the corresponding post mitochondrial supernatant (approximately 200 µg) were aliquoted into the appropriate 1.5 ml microfuge tubes (A-E) and the volume made up to 20 µl with 0.6 M mannitol/ 20 mM HEPES pH7.4 where necessary. Proteinase K and Triton X-100 were added as shown below and Tubes were incubated for 15 minutes at 0^0C after which 20 µl SDS sample buffer was added and tubes boiled for 3 minutes. The contents of each tube were then subjected to 10% SDS-PAGE and proteins transferred to a nylon membrane. Western blots were probed with antiserum raised against mature cytochrome b_2 and visualised using HRP conjugated IgG (Methods 2.12.)

Key: A = Supernatant.

B = Supernatant + 100 µg/ml (final conc.) proteinase K.

C = Mitochondria.

D = Mitochondria + 100 µg/ml proteinase K.

E = Mitochondria + 100 µg/ml proteinase K + 0.3% Triton X-100.

In order to address this question the modified *CYB2* gene encoding the precursor lacking amino acids 2-32 was cloned into pRC23 and the resulting recombinant vector, pRC404, transformed into *E.coli* strain POP2136. *E.coli* cells transformed with pRC401, pRC403 (chapter 3.4) and pRC404 were grown at 30^0C until $O.D._{600}$=0.3 at which point gene expression was induced for 90 minutes and protein samples from each transformant prepared as described in Methods 2.15. The western blot in figure 4.11 shows that intermediate sized cytochrome b_2 is accumulated in *E.coli* cells harbouring pRC404 in amounts greater than that of precursor but less than those of mature cytochrome b_2 90 minutes after induction in the coresponding transformants.

4.10. Effect of expression of intermediate sized cytochrome b_2 on *E.coli* cell growth.

The growth rate of *E.coli* cells expressing precursor, intermediate and mature-sized cytochrome b_2 was monitored after the induction of gene expression. Figure 4.12 shows that as previously described in chapter 3, cells exhibit a transient lag in growth upon induction of precursor expression whereas cells expressing mature protein grow as normal. Cells expressing intermediate-sized protein however stop growing approximately 60 minutes after the induction of gene expression. This inhibition of growth was observed to continue for several hours.

4.11. Subcellular localisation of intermediate sized cytochrome b_2 in *E.coli*.

E.coli expressing precursor, intermediate and mature sized cytochrome b_2 were subfractioned as described in Methods 2.16. Proportionate amounts of each subcellular fraction were electrophoresed on a 10% polyacrylamide gel and western blots performed (Methods 2.12). Figure 4.13 shows the distribution of intermediate sized cytochrome b_2 in comparison to that of the precursor and mature forms in *E.coli*. The degree of cross contamination between fractions and the relative abundance of each protein within a particular fraction is shown in

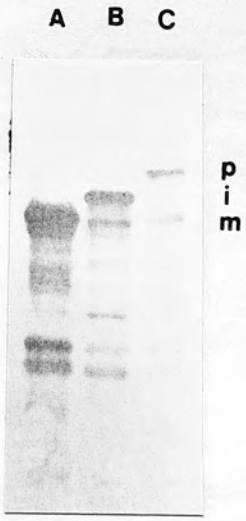

Figure 4.11.

Western blot of E.coli proteins from cells transformed with pRC403 (A), pRC404 (B) and pRC401 (C).

POP2136 cells transformed with the above constructs were grown and induced for 90 minutes. Whole cell protein extracts were prepared from each transformant and approximately 10 μg subjected to SDS-PAGE (10 % gel). Proteins were then transferred to a nylon membrane and western blots were probed with antiserum raised against mature cytochrome b_2 and visualised using HRP conjugated IgG-(Methods 2.12.)

Key: p = precursor, i = intermediate, m = mature.

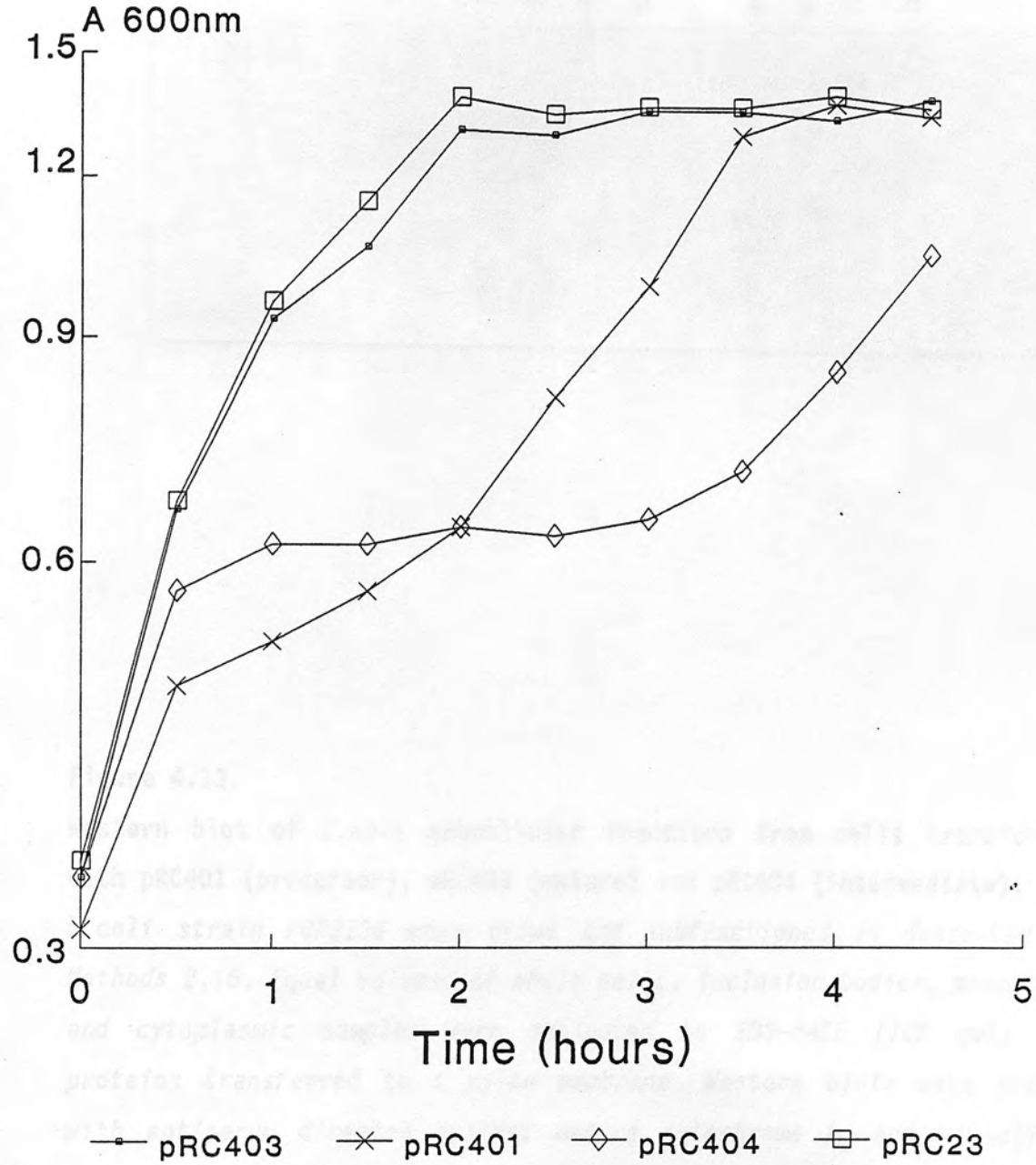

Figure 4.12.

Growth of *E.coli* strain POP2136 transformed with pRC23, pRC401 (precursor), pRC403 (mature) and pRC404 (intermediate).

Transformants were grown on LB + ampicillin at 30°C to an $O.D._{600}$=0.3. Cultures were shifted to 42°C and growth monitored at the indicated time points.

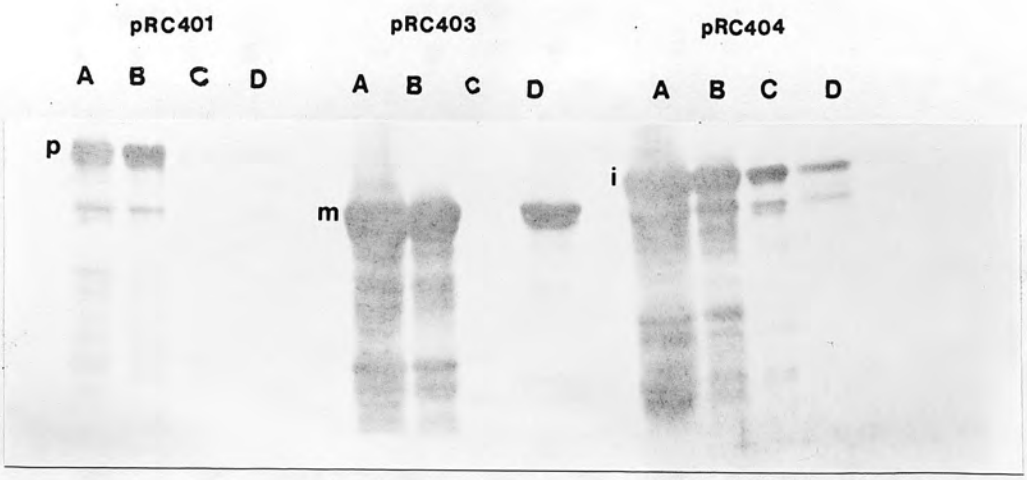

Figure 4.13.

Western blot of *E.coli* subcellular fractions from cells transformed with pRC401 (precursor), pRC403 (mature) and pRC404 (intermediate).

E.coli strain POP2136 were grown and subfractioned as described in Methods 2.16. Equal volumes of whole cells, inclusion bodies, membranes and cytoplasmic samples were subjected to SDS-PAGE (10% gel) and proteins transferred to a nylon membrane. Western blots were probed with antiserum directed against mature cytochrome b_2 and visualised using HRP-conjugated IgG (Methods 2.12.)

Key: A = Whole cells, B = Inclusion bodies, C = Membranes, D = Cytoplasm.

p = precursor, i = intermediate and m = mature.

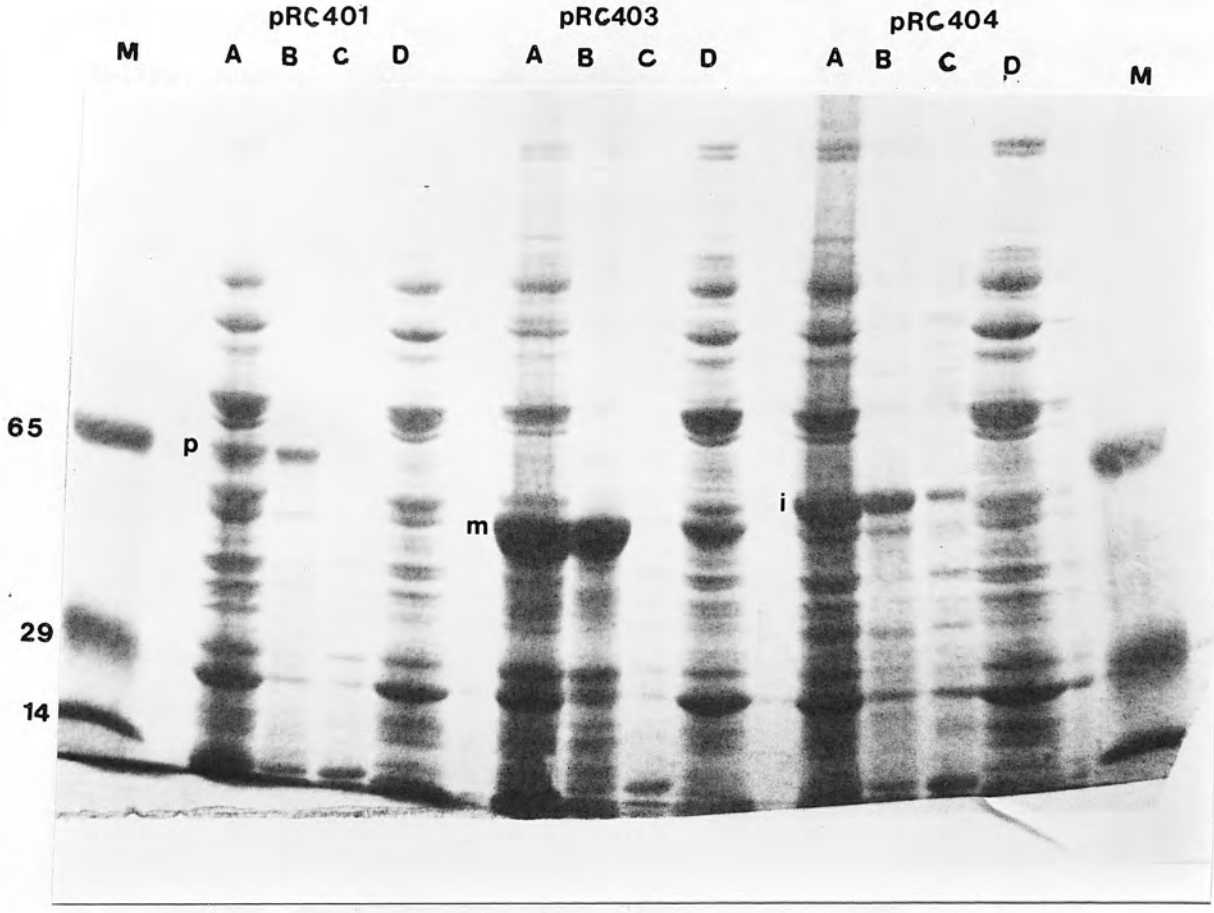

Figure 4.14.

Coomassie Blue staining of *E.coli* subcellular fractions from cells transformed with pRC401 (precursor), pRC403 (mature) and pRC404 (intermediate).

Fractions were prepared as described in Methods 2.16. Equal volumes of protein were subjected to SDS PAGE (10% gel) and gels stained with Coomassie Blue (Methods 2.11.)

Key: M = Molecular weight markers, A = Whole Cells, B = Inclusion bodies, C = Membranes, D = Cytoplasm.

p = precursor, i = intermediate and m = mature.

figure 4.14 where gels have been stained with Coomassie Blue. These results show that in *E.coli* cells transformed with pRC404, intermediate-sized cytochrome b_2 accumulates mainly as inclusion bodies, however a proportionately greater amount of this intermediate form is found in the membrane fraction than either the precursor or mature form. Although only a relatively small proportion of the total intermediate protein expressed is associated with the membrane fraction it is sufficient to be detectable upon Coomassie Blue staining and constitute a significant fraction of the total membrane protein (figure 4.14) It was not known whether this association of the intermediate sized cytochrome b_2 with *E.coli* membranes reflects the anchoring of the protein in either the inner or outer cell membrane, since total *E.coli* membranes are recovered in the subfraction method used here, or whether the protein has only a peripheral association.

4.12. Discussion.

Pratje and Guiard (1986) showed that the second proteolytic cleavage of the cytochrome b_2 precursor during its transport into the mitochondrial intermembrane space was mediated by a sequence-dependent protease. This result was used here to construct a mutant form of the polypeptide which would hopefully not be a substrate for the protease catalysing the second cleavage step. Alteration of one of the amino acid residues at the second cleavage site from a glutamate to a lysine residue results in the inhibition of processing at this site and leads to the accumulation of an intermediate sized cytochrome b_2. This inhibition appears not to be complete as some mature sized protein is apparent in western blots but this represents only a minor fraction of the total cytochrome b_2. As expected the intermediate form is located in the mitochondrial inner membrane as determined by mitochondrial subfractionation and western blotting. Much of the wild type mature cytochrome b_2 is present not just in the intermembrane space but in all other mitochondrial subfractions as well. Great difficulty was encountered in achieving efficient separation of mitochondrial

subfractions such that cytochrome b_2 could be found solely in the intermembrane space. The intermembrane space contents were released using the method of Daum *et al.* (1982b) i.e. by controlled osmotic shock. Mitoplasts were then recovered by centrifugation leaving the soluble intermembrane space components in the supernatant. Not all mature cytochrome b_2 is released during this osmotic shock. This could conceivably have been achieved by a further decrease in osmolarity but since such a step results in the release of proteins from the mitochondrial matrix, it is impossible to completely separate these two subfractions. The presence of mature cytochrome b_2 in the post mitochondrial supernatant reflects the leakage of some intermembrane space contents probably due to mechanical damage to the outer membrane during preparation of mitochondria. In contrast, intermediate cytochrome b_2 is detected only in the membrane fraction and this association presumably prevents the leakage of the protein into other compartments during subfractionation.

The intermediate form of cytochrome b_2 was previously observed to exhibit full enzyme activity when measured in samples of mitochondria or mitoplasts either using cytochrome c or ferricyanide as the electron acceptor. The activity of both mature and intermediate forms of cytochrome b_2 was also measured in samples of mitochondria in this study. In contrast to earlier observations, (Pratje and Guiard, 1986) intermediate cytochrome b_2 was found only to exhibit full enzyme activity with ferricyanide as the electron acceptor. When using cytochrome c, the natural electron acceptor, the specific activity was approximately only 50% that of the mature enzyme. This disparity in the specific activity of each form of the enzyme was reproducibly observed in several different preparations of mitochondria and was independent of the concentration of cytochrome c present in the assay. One possible explanation for this observed difference could be the limited access of cytochrome c to the active site of the cytochrome b_2 molecule due to the anchoring of the N-terminus in the inner mitochondrial membrane. It is not known whether the intermediate form assembles into tetramers

like the mature enzyme or indeed whether monomers themselves are active but it is conceivable that membrane localisation might restrict access of cytochrome c without affecting access of the much smaller ferricyanide ion. The disparity between the specific activity of mature cytochrome b_2 when using both cytochrome c and ferricyanide as electron acceptors may be due to the ionic strength of the assay buffer. Lederer (unpublished) has recently reported that increasing the ionic strength of the assay buffer results in similar levels of specific activity.

Mitochondria in which intermediate cytochrome b_2 had accumulated were used as a starting point for purification of this protein prior to amino acid sequencing. Intermediate cytochrome b_2 could be partially extracted from the inner membrane in buffers containing 1% Triton X-100 and 200 mM KCl. Upon precipitation of proteins with acetone however, intermediate cytochrome b_2 could not be redissolved even in buffers containing the detergent. SDS-PAGE of the partially purified intermediate and Coomassie Blue staining showed the relative proportion of intermediate cytochrome b_2 in this fraction. Several attempts were made to electroelute the protein from such stained gels using a Biotrap (Schleicher and Schuell Ltd.). This gave poor recovery and was further limited by the availability of suitable buffers in which to perform the electroelution step. Attempts to sequence material prepared in this way were unsuccessful. These problems were overcome by electrophoretic transfer of proteins onto PVDF membrane after SDS-PAGE. Proteins were then stained with Coomassie Blue and the bound intermediate cytochrome b_2 introduced into the sequenator.

The alignment of the amino acid sequence derived for the intermediate N-terminus with that previously deduced for the complete precursor (Guiard, 1985) allows the position of the first cleavage site to be located to between Tyrosine-32 and glycine-33. Thus the first 32 amino acids of the precursor are removed in the mitochondrial matrix by the chelator-sensitive protease. The primary cleavage site had previously been suggested to exist one amino acid proximal to the site determined above based on comparison of a number of known matrix protease cleavage

sites (Hartl *et al.*, 1987). Nicholson and Neupert (1988) have compared 17 cleavage sites known to be recognised by the matrix protease but report a low degree of sequence similarity. The amino acid at position -1 (with respect to the site of cleavage) is usually uncharged and very often hydrophobic in lower eukaryotes while the residue at position -2 possesses an aliphatic or positively charged side chain. Charged amino acids at position +1 and +2 are rare with serine residues common. The first cleavage site on the cytochrome b_2 precursor therefore exhibits the albeit weakly conserved features common to other known matrix protease cleavage sites.

After the removal of the first 32 residues from the N-terminus of the cytochrome b_2 precursor by the matrix protease the remaining 48 amino acids of the presequence are then proposed to re-target the intermediate form back across the mitochondrial inner membrane into the intermembrane space (Hartl *et al.*, 1987). What is the nature of the targeting information within the intermediate presequence?

Analysis of the remaining 48 amino acids of the cytochrome b_2 presequence reveals a number of features of potential importance to its targeting. The first 20 amino acids contain 5 positively charged residues, 4 serine residues and 3 threonine residues in no obvious arrangement. These features alone are common to matrix targeting sequences in general, ie sequences responsible for targeting from the cytoplasm into the mitochondrial matrix. Since the first 32 residues of the presequence have already been proposed to contain the information for matrix localisation, the existence of a second similar sequence might appear to represent the unnecessary duplication of targeting information. In addition to a potential matrix targeting signal the intermembrane space presequence also contains a series of 20 uncharged residues (punctuated by a single arginine). Similar uncharged sequences have been identified in the presequences of other processing intermediates destined for the mitochondrial intermembrane space and this has led to the proposal that such sequences represent targeting signals already present in the prokaryotic ancestor of mitochondria

(Hartl *et al.*, 1987). The similarity between the uncharged regions of intermediate presequences and bacterial signal sequences is regarded as supportive of this notion.

It would appear then that the presequence responsible for re-export of intermediate cytochrome b_2 contains sequence elements associated with both mitochondrial and bacterial membrane translocation. Whether these sequences were able to effect either targeting response *in vivo* was examined in experiments performed in the second half of this chapter. When intermediate-sized cytochrome b_2 is synthesised on cytoplasmic ribosomes, a fraction is targeted to the mitochondrion despite the absence of the matrix targeting signal in the first 32 amino acids of the presequence. The targeting of the intermediate to the mitochondrion was less efficient than that of the precursor with approximately 50% of the total intermediate present in the cytoplasm. Recently, several mitochondrial proteins have been shown to be capable of being imported into mitochondria *in vivo* even in the absence of the entire presequence (Volland and Urban-Grimal, 1988; Thompson and McAllister-Henn, 1989), however work carried out in our laboratory has shown that when mature cytochrome b_2 is synthesised on cytoplasmic ribosomes no association with mitochondria is observed (R.Pallister-Ph.D. thesis, in preparation). Therefore the targeting of intermediate cytochrome b_2 to mitochondria, albeit with reduced efficiency, would appear to be dependent on the intermediate presequence.

In addition to localisation of intermediate cytochrome b_2 in the yeast cell by western blot analysis, whole cell, mitochondrial and post mitochondrial supernatant fractions were analysed for cytochrome c reductase activity. When measured in whole cell extracts the specific activity of intermediate sized protein was approximately 50% that of the wild-type enzyme. Surprisingly, this activity was observed to decrease during catalysis indicating that the intermediate sized enzyme loses activity during enzyme turnover. White *et al.* (1989) have recently reported that a mutant form of cytochrome b_2 lacking the C-terminal tail similarly exhibits deactivation kinetics. In this case

the flavin prosthetic group appears to be lost as a result of sustained enzymatic turnover. Similar results were observed when the activity of purified mitochondrial fractions was assayed but only a very low level of cytochrome c reductase activity was observed in post-mitochondrial supernatant fractions despite the fact that western blot analysis indicates that approximately 50% of the intermediate protein remains in the cytoplasm. The significance of these observations is not known but one reason for the lack of observed enzyme activity in cytoplasmic fractions could be due to the association of soluble proteins with the intermediate cytochrome b_2 which normally are required for import and may alter the tertiary structure accordingly.

Although the intermediate cytochrome b_2 is targeted to the mitochondrion, it is not however imported to a protease protected form indicating that the protein remains bound at the mitochondrial surface. This failure of the intermediate to be imported into the mitochondrion presumably accounts for the absence of its processing to the mature form.

The observed targeting of intermediate-sized cytochrome b_2 contrasted to the previous study findings of Riezman *et al.* (1983a) who showed that intermediate cytochrome b_2 did not bind to isolated mitochondria *in vitro*. Recently, however Pfanner *et al.* (1988b) have pointed out that that the apparent targeting of certain proteins to mitochondria need not reflect the correct interaction with each component of the import machinery but could occur, at lower levels, via a bypass mechanism, especially when a protein is present in artificially high amounts, as is the case when proteins are expressed on high copy number vectors.

Other factors prevent the positive identification of a second mitochondrial targeting signal within the intermediate cytochrome b_2 presequence. Firstly, although the intermediate presequence contains a number of randomly distributed positive charges these need not enable the formation of an amphiphilic structure known to be required for efficient mitochondrial targeting (Allison and Schatz, 1987). Secondly,

the existence of 2 negatively charged amino acid residues within the putative presequence is not consistent with the general requirements of mitochondrial presequences.

It therefore cannot be stated categorically that the observed targeting of intermediate cytochrome b_2 to mitochondria as indicated in these experiments is a result of a second matrix targeting signal within the intermediate presequence.

As previously discussed, the intermediate presequence also contains a series of uncharged residues which resemble the hydrophobic core of a prokaryotic signal peptide. Does intermediate cytochrome b_2 contain a funtional bacterial export signal? To address this question the intermediate sized cytochrome b_2 was expressed in the bacterium *E.coli*. Like both the precursor and mature proteins, the intermediate can be stably expressed in *E.coli* cells. However unlike expression of the precursor or mature form, expression of intermediate sized cytochrome b_2 in *E.coli* results in the inhibition of cell growth. This inhibition of cell growth was observed to persist for several hours after the induction of gene expression beyond which the $O.D._{600}$ of cultures expressing intermediate cytochrome b_2 increased at a normal rate to stationary phase. When plasmid DNA from cultures which had apparently overcome this inhibition of cell growth was analysed by restriction digestion it was apparent that a rearrangement of plasmid DNA had occurred. Thus the re-establishment of growth after inhibition due to the expression of intermediate cytochrome b_2 probably represents growth of a subpopulation of cells which no longer express this protein

The observed inhibition of cell growth upon expression of intermediate cytochrome b_2 is reminiscent of the expression of *malE-lacZ* hybrids in *E.coli* (Bassford *et al.*, 1979). When an amino terminal portion from the periplasmic maltose binding protein was fused to β–galactosidase the resultant chimeric protein was observed to be targeted to the cytoplasmic membrane when expressed in *E.coli*. The protein was not however completely translocated into the periplasm but remained associated with the cytoplasmic membrane such that the export of other

periplasmic and membrane proteins was blocked. As a consequence *E.coli* cells expressing such *mal*E-*lac*Z fusions stopped growing and the cells died. These results indicated that several proteins shared a common site of export in *E.coli*.

The inhibition of cell growth in response to expression of intermediate cytochrome b_2 in *E.coli* might therefore be due to its association with the cytoplasmic membrane and the resulting interference with normal protein export. To investigate this possibility *E.coli* cells expressing precursor, intermediate and mature forms of cytochrome b_2 were subfractionated and the location of each form of the protein examined by western bloting. All forms of the protein are found predominantly as inclusion bodies within the cell. As discussed in chapter 3.7., this finding may be a result of high level overexpression of each form of the protein. A smaller but significant fraction of the intermediate form is however associated with the membrane fraction. Staining of fractions with Coomassie Blue reveals that a protein with molecular weight similar to that of the intermediate form constitutes a significant fraction of the total membrane associated protein in cells expressing the intermediate form. In the experiment presented in figures 4.13 and 4.14 of this chapter, the total levels of membrane protein were low compared with other subcellular fractions, in addition the membrane fraction from cells expressing intermediate cytochrome b_2 contained slightly more protein than from the other transformants. While this fact prevented a true comparison between the amounts of each cytochrome b_2 species associated with membranes, the relative amount of the intermediate form in this fraction was greater than that of precursor or mature.

If intermediate cytochrome b_2 is targeted to the membrane in *E.coli* cells it is unlikely that all the protein synthesised during the given period of induction could be assimilated into or associated with the membrane owing to the limited space available. The location of the majority of the expressed intermediate form in inclusion bodies might therefore represent an excess of protein after maximal association with

the membrane fraction. Alternatively, the misfolding of the majority of the intermediate form due to its high level expression (see chapter 3 discussion) might allow only a proportion of the total amount of this protein to assume a conformation compatible with its targeting to the cell membrane.

Further experiments are required to confirm the specific association of intermediate cytochrome b_2 with *E.coli* cell membranes (see chapter 5), but from the evidence presented in this chapter it would appear at least that this form of the protein interacts with components of *E.coli* cells with which the corresponding precursor and mature forms do not.

Does the apparent targeting of intermediate cytochrome b_2 to the *E.coli* cell membrane reflect the conservation of a prokaryotic export signal in this protein? Unlike the intermembrane space proteins cytochrome c_1 and the Rieske Fe/S protein from the ubiquinol:cytochrome c complex, both of which share extensive homologies with analogous proteins from *R.sphaeroides*, yeast cytochrome b_2 does not exhibit such an obvious similarity to any bacterial proteins. Indeed the properties of lactate dehydrogenase enzymes from different bacteria, which are NAD-dependant dehydrogenases, are themselves quite diverse (Tarmy and Kaplan, 1968). The requirement of a series of uncharged residues for re-export of a protein from the matrix is analogous to the requirement of a similar sequence in the routing of proteins into the thylakoid lumen in chloroplasts. Smeekens *et al.* (1986) has shown that plastocyanin from *Silene pratensis* is synthesised as a precursor with a 66 amino acid N-terminal transit peptide. Gene fusion studies have shown that the extreme N-terminal 30-40 residues are responsible for targeting into the chloroplast stroma (analogous to the mitochondrial matrix). The proteolytic removal of this stromal targeting sequence generates an intermediate form of the protein which is directed to the thylakoid membrane by the remaining 30-35 residues which constitute the intermediate presequence. This intermediate presequence contains a series of 20 uncharged residues rich in valine and alanine analogous to the series of uncharged residues in the intermediate cytochrome b_2

presequence. Recently, the intermediate presequence from another thylakoid protein the 33 kDa subunit from the oxygen evolving complex, was shown to contain sufficient information for the thylakoid localisation of a non chloroplast protein (Ko and Cashmore, 1989).

In summary although a series of uncharged amino acid residues would appear to be required for targeting of processing intermediates from the mitochondrial matrix to the intermembrane space and, by analogy, from the stroma to the thylakoid membrane of chloroplasts, this sequence may only represent the conservation of a prokaryotic export signal in proteins which also exhibit a strong degree of homology in function, structure and topology with similar bacterial proteins. In proteins such as cytochrome b_2 the intermediate presequence may have evolved independently possibly to acquire additional elements to enable interaction with the mature protein structure.

CHAPTER 5.

SUMMARY AND PERSPECTIVES.

Knowledge of how proteins are imported into mitochondria has advanced rapidly during the last 10 years. While some of the features associated with import are recognised, many of the molecular mechanisms are unclear and it is probable that many components involved remain to be identified. This study has attempted to develop two areas of the field of mitochondrial protein import;

(i) By isolation and characterisation of the precursor of cytochrome b_2 a mitochondrial intermembrane space protein.

(ii) By an examination of membrane targeting signals within the bipartite presequence of this mitochondrial precursor.

Initial experiments aimed at accumulating precursor protein prior to its purification were carried out in yeast. Import of proteins into mitochondria in yeast cells occurs rapidly and under normal conditions pools of precursor proteins can only be detected by sensitive methods. Using a multi-copy vector to express the *CYB2* gene, it was possible to saturate the processing of the cytochrome b_2 precursor, some of which was bound to the mitochondrial surface and hence to increase the ratio of precursor to mature protein within yeast cells. A further slight increase in this ratio was observed upon growing cells in the presence of an uncoupler of oxidative phosphorylation but even after this treatment the overall level of precursor was still small compared with that of mature protein.

In contrast, expression of the *CYB2* gene in *E.coli* resulted in the accumulation of the unprocessed precursor of cytochrome b_2 to levels up to 3% of total cell protein. Interestingly, mature cytochrome b_2 was much more efficiently expressed in an identical system in *E.coli* than the corresponding precursor. The significance of this result is not fully understood but it may be due to the interaction between a cellular component and the precursor protein.

The cytochrome b_2 precursor formed insoluble aggregates or inclusion bodies when expressed in *E.coli*. This insolubility presented both advantages and disadvantages for the subsequent purification of precursor from *E.coli* cells. Firstly, aggregation probably prevents excessive proteolytic degradation of precursor within the cell. Secondly, providing cells were efficiently lysed, the inclusion body fraction could be easily purified by centrifugation representing approximately a 20 fold enrichment of precursor. Subsequent purification of this fraction was however hindered by its lack of solubility. Use was made in this study of 8 M urea to denature the precursor and effect its solubilisation. The solubilised inclusion body fraction was then subjected to anion exchange chromatography using an FPLC system but this failed to effectively resolve the precursor from contaminating proteins.

A large number of proteins which form inclusion bodies when expressed at high level in *E.coli* have been purified after denaturation, with subsequent renaturation being accompanied by the restoration of biological activity, (for review see Marston, 1986). Crooke and Wickner (1987) have used affinity chromatography followed by anion exchange to purify pro-Omp A from *E.coli* in the presence of 8 M urea. Upon removal of the urea the purified protein retained its ability to insert into lipid vesicles. These experiments highlight the potential for the purification of membrane proteins whose hydrophobicity might make purification by conventional means difficult.

Preparation of milligram quantities of the cytochrome b_2 precursor would enable the examination of several features of mitochondrial precursor proteins. Endo and Schatz (1988) have recently used a purified fusion protein in which the COXIV presequence is attached to the N-terminus of DHFR to conduct such studies on precursor folding. These authors reported that attachment of a mitochondrial presequence to DHFR did not significantly alter its three dimensional structure and the fusion protein still retained near normal DHFR enzymatic activity. A considerable amount of experimental evidence however exists to

suggest that in both mitochondrial and bacterial precursors a presequence can at least slow the kinetics of folding of the attached polypeptide and may even induce a permanent conformational difference (Oxender *et al.*, 1982; Chien and Freeman, 1986; Park *et al.*, 1988). Clearly further study into the structure of authentic mitochondrial precursors is therefore necessary to resolve these conflicting observations.

The accumulation of intermediate cytochrome b_2 in yeast cells expressing a modified form of the *CYB2* gene enabled sufficient quantities of this form to be extracted and an N-terminal amino acid sequence and thus the primary processing site, to be determined. This cleavage site defines the beginning of a 48 amino acid targeting signal at the N-terminus of the intermediate form which has been proposed to direct the protein from the mitochondrial matrix to the intermembrane space (Hartl *et al.*, 1987). This targeting signal apparently contained two different sequence elements, an N-terminal domain which displayed some of the features associated with mitochondrial (matrix) targeting signals and a C-terminal domain consisting of a series of uncharged residues reminiscent of a bacterial signal peptide which the above authors have proposed represents the conservation of an export signal from the endosymbiotic ancestor of mitochondria.

The experiments described here show that when expressed in yeast cells, intermediate cytochrome b_2 is targeted to mitochondria albeit at a lower efficiency than the precursor. However, intermediate cytochrome b_2 remained bound at the outer face of the mitochondrion and was not imported into the organelle. When intermediate cytochrome b_2 was expressed in *E.coli* the cells stopped growing. This effect i.e. complete inhibition of growth, was specific to the expression of the intermediate form since expression of mature cytochrome b_2 had no effect on cell growth rates while the expression of the complete precursor produced only a transient reduction in growth rate.

The majority of all three forms of cytochrome b_2 when expressed in *E.coli* formed insoluble inclusion bodies although a significant

fraction of the mature form remained soluble in the cytoplasm. In addition the subfractionation experiments described in chapter 4 of this study showed that a proportionately greater amount of intermediate cytochrome b_2 was present in the membrane fraction than either the precursor or mature forms. While these experiments did not provide conclusive proof that the intermediate form of cytochrome b_2 was targeted to the membrane when expressed in *E.coli*, the results suggest that it might participate in molecular interactions possibly with components of the protein export machinery that the corresponding precursor and mature proteins do not.

Future work would certainly be aimed at improving the quality of subfractionation data to determine the localisation of each form of cytochrome b_2 in *E.coli*. A first step towards achieving this would be a reduction in the levels of expression of these proteins possibly by use of an alternative expression vector with a less efficient promoter. This should eliminate the problem of cross-contamination of subcellular fractions as a result high over expression. Should such experiments confirm the association of intermediate cytochrome b_2 with E.coli membranes the nature of such an interaction could be further characterised e.g. is the association peripheral i.e. disruptable in the presence of high salt concentrations, or is the intermediate form anchored in the lipid bilayer of the inner or outer membrane of *E.coli*? If the intermediate form of cytochrome b_2 does contain a functional bacterial targeting signal it would be interesting to determine whether the intermediate forms of other intermembrane space proteins possess a similar signal. In addition if re-translocation of proteins from the mitochondrial matrix to the intermembrane space does rely on ancestral protein targeting signals then other components of this pathway might similarly show homologies to bacterial counterparts. One such candidate could be the protease responsible for the second proteolytic processing step of cytochrome b_2 which also catalyses the processing of the mitochondrially encoded COXII protein. This protein which has not been fully characterised is functionally equivalent to bacterial leader

peptidase. The characterisation and subsequent comparison of this protein with leader peptidase could reveal new evidence for the maintainence of an ancestral protein targeting pathway in the import of proteins to the intermembrane space of mitochondria.

In addition to the areas covered in this study, several aspects of protein targeting in general remain active areas of research. Although much work has been done to characterise some of the biochemical features of targeting signals, little is known of how these sequences interact with other components of the translocation apparatus both in the cytoplasm and at the membrane surface. The ability to isolate purified proteins and study their interaction *in vitro* will undoubtedly be of great importance in elucidating the nature of such interactions. Recent studies by Lecker *et al.*, (1989) have begun to examine the interaction of bacterial precursor proteins with several purified chaperonins but these workers acknowledge the need for further work to characterise the interactions involved.

A further key area of research is likely to be in examining exactly how proteins traverse the lipid bilayer. In particular, the identification and characterisation of translocator proteins is a major goal although the existence of such proteins in any system has yet to be conclusively shown. One approach which may prove useful in identifying such proteins is through the photo-crosslinking of a modified precursor protein to a component of the membrane through which it is translocated. This method has already been used to identify a component of the mitochondrial outer membrane shown to be involved in protein uptake by the organelle (Vestweber *et al.*, 1989). A similar strategy has been employed in the identification of a 35 kDa glycoprotein in microsomal membranes which might represent a transmembrane channel component (T.Rapoport, results presented at 19th FEBS meeting, Rome 1989). A further possibility concerning the translocation of proteins across lipid bilayers remains that proteins cross the membrane at regions where the lipids have been rearranged from the typical bilayer

configuration, the energy for such rearrangements coming possibly from ATP hydrolysis.

The emerging biochemical data from studies addressing the above questions can be reinforced by parallel genetic studies. Hitherto a relatively small number of genes linked to mitochondrial import have been positively identified. However, several studies are currently underway and their results may help to unravel some of the mechanisms involved in different protein targeting pathways.

APPENDIX A.

Sequences of oligonucleotides.

Name	Sequence
075A	TAGACAACTCAATTCATGGAGCCGAA
B2-1	CCGGAATGCTAAAATAC
K81	TAGACAACAAGCCGAA
079D	CTTAAGGCCTTACCCAAGATGGCAAG
102A	CTGCACAATATTTCAAGC

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